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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Our laboratory has recently identified a new protein, Pin1, that is involved in checkpoint control. Pin1 interacts with mitotic phosphoproteins and helps to orchestrate the timing of mitotic events. We found that Pin1 is highly overexpressed in breast cancer. Pin1 levels correlate with the levels of cyclin D1 protein as well as with cyclin D1 mRNA levels in human breast tumors. We have shown that Pin1 is a transcriptional acitvator of cyclin D1. Activation occurrs indirectly through the binding of phosphorylated c-jun. Our data indicate that Pin1 may contribute to neoplastic transformation by causing accumulation of cyclin D1. In addition, Pin1 contributes to cyclin D1 overexpression by regulating the turnover and subcellular localization of beta-catenin and inhibiting its interaction with APC. In Pin1 knock-out mice, mammary epithelial cells fail to undergo the usual proliferative burst during pregnancy, a phenotype that is very similar to the cyclin D1 knock-out. Finally, the PIN1 gene itself is an E2F target gene and essential for Neu/Ras induced transformation of mammary epithelial cells.

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Role of the Prolyl Isomerase Pin1 in Oncogenesis
Gerburg M. Wulf

I. Introduction

Phosphorylation of proteins on serine or threonine residues preceding proline (Ser/Thr-Pro) is a major intracellular signaling mechanism. The peptidyl-prolyl cis-trans isomerase Pin1 has recently been shown to isomerize phosphorylated Ser/Thr-Pro motifs in a specific subset of phosphoproteins. This "post-phosphorylation" isomerization leads to conformational changes in the substrate protein and modulates their functional properties. Thus, Pin1 may provide a new post-translational level of control that orchestrates the general increase in serine-phosphorylation and results in an organized set of mitotic events.

Early on Pin1 had been shown to interact with a wide range of mitotic phosphoproteins, and the presence of Pin1 is essential for progression through mitosis. Recently, overexpression of Pin1 was found in a high percentage of human cancers, most exhaustively studied in breast cancer. In cancer cells, Pin1 regulates the expression of cyclin D1 through (a) cooperation with Ras signaling (b) inhibiting the interaction of beta-catenin with APC and (c) direct stabilization of the cyclin D1 protein. In addition, we have recently identified Pin1 itself as a downstream target of oncogenic Ras/Neu signaling via activation of the E2F transcription factor.

II. Body
Task 1 (months 1 – 12)
<u>To examine whether Pin1 or Pin2 are candidate tumor markers for breast cancer</u>

Given that Pin1 regulates the conformation of certain phosphorylated Ser/Thr-Pro motifs, we first asked whether Pin1 expression is aberrantly regulated in human breast cancer. Initially we examined the levels of Pin1 expression in normal and neoplastic breast tissues. In 10 normal and 51 primary human breast cancer tissues examined, we observed striking differences in the levels of Pin1 protein expression. 71 % of grade II tumors and 90 % grade III tumors overexpressed Pin1. Although we observed considerable inter-individual variations, especially in grade II and III tumors, the mean expression level of Pin1 was about 10 times higher in cancer samples than in the normal controls. Pin1 levels positively correlated with the tumor grade in invasive breast cancer, as determined by Kruskal Wallis test. For further details please see reference 1. Given the striking expression data we analyzed whether Pin1 expression correlated with any known breast cancer marker. It turned out that Pin1 levels tightly correlated with cyclin D1 levels. There was a positive correlation with Her2/neu expression that did not reach statistical significance because of the small number of samples. Consistent with the tight correlation between Pin1 and cyclin D1 observed in human breast cancer samples, Pin1 positively regulates cyclin D1 function at the transcriptional level in collaboration with several different oncogenic signaling pathways and also through post-translational stabilizatio (1, 2, 3, 6).

Pin1 binds phosphorylated c-Jun on Ser 63/73-Pro motifs and thereby increases its transcriptional activity towards the cyclin D1 promoter via the AP-1 site. The AP-1 complex regulates a wide range of cellular processes, including cell proliferation, cell death, survival and differentiation. Pin1 binds c-Jun, that is phosphorylated on Ser 63 and 73, and also increases its ability to activate the cyclin D1 promoter in cooperation either with activated JNK or oncogenic Ha-Ras. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun, indicating that endogenous Pin1 is also required for the optimal activation of c-Jun. Thus, Pin1 is a potent modulator of

phosphorylated c-Jun in inducing cyclin D1 expression, presumably by regulating the conformation of the phosphorylated Ser-Pro motifs in c-Jun. These studies on the biochemical consequences of Pin1 overexpression were performed in addition to what was proposed in Task 1, and this change in strategy was prompted by the clinicophathological data, i.e. the close correlation of Pin1 overexpression with cyclin D1.

The extraction of genomic DNA from the archival tissues for Southern blotting for telomer length or amplification of the *PIN1* gene met with unexpected difficulties: The quality and quantity of the DNA isolated from the tissues that were up to 15 years old was not sufficient for Southern blot analysis. Therefore, these analyses will be performed using a different tissue bank, we will try to do this through the SPORE breast cancer grant sponsored tissue bank at the Harvard Cancer Center.

Pin2 levels in the 50 tumor specimen were significantly lower then in the 10 normal specimen examined, however there was no correlation with tumor grade, LN status of the patient, Cyclin D1 or PCNA or Estrogen Receptor Status. All the pertinent results are presented in the attached paper published in Oncogene (5). The interaction of Pin1 and Pin2 with the tumor suppressor nm23 has not yet been pursued, because we gave the analysis of the oncogenic properties of Pin1 priority. I do want to add, though, that an independent yeast two hybrid screening performed by Dr. Xiao Zhou in our laboratory, has confirmed the interaction of Pin2 with nm23. Work on this task will be started soon.

Task 2 (months 12-36)
To analyze the role that Pin1 and Pin2 play in neoplastic transformation *in vitro* and *in vivo*

Several MCF7 cell lines inducible for Pin1 had been established. Inducibility had been verified by Western Blotting (see Fig. 3 in reference 1). The induction of Pin1 caused an increase in cyclin D1 expression. However, it turned out that additional (transgenic) Pin1 did not affect the behavior of these cells in the matrigel assay. Also cell cycle ditribution and growth pattern remained unaffected. In essence, it was not possible to alter an already transformed phenotype through an increase in Pin1 levels. Therefore, we resorted to immortalized human mammary epithelial cells that are not yet transformed. We stably transfected GFP-Pin1 and control GFP into MCF-10A cells, a spontaneously immortalized, but non-transformed mammary epithelial cell line that has been widely used for cell transformation studies. Multiple stable cell lines were obtained that had similar properties (3). Consistent with our previous studies (1, 2), cyclin D1 protein levels were elevated in these GFP-Pin1 stable clones as compared with control GFP cells, with cyclin D1 levels correlating with exogenous Pin1 expression levels (Fig. 5A of ref 3). Although there was no detectable difference in cell morphology and growth rate on plastic plates between GFP-Pin1 and control GFP cell lines, overexpression of GFP-Pin1, but not GFP, conferred anchorage-independent cell growth in soft agar (Fig. 5B of ref 3). However, the size and frequency of colonies were much less than those of Neu/Ras-transformed MCF-10A cells (Fig. 5B vs Fig. 6F of ref 3). Moreover, like parental MCF-10A cells, GFP-Pin1 stable cell lines were unable to survive in DMEM media supplemented with10% fetal bovine serum (data not shown), while Neu/Ras-transformed MCF-10A cells can grow normally in this medium (Fig. 6C of ref 3). These data suggest that although overexpression of Pin1 appeared to be insufficient to fully transform MCF-10A cells, it might trigger some early events of cell transformation.

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To further investigate this possiblility, we performed a three-dimensional cell differentiation assay using exogenous basement membrane matrix (Matrigel). We found that GFP-expressing cells formed acini with basally polarized nuclear organization, intact cell-cell junctions and visible lumina inside, as indicated by immunostaining with antibodies against the cell-cell junction marker E-cadherin and with the DNA dye TOPRO, followed by confocal microscopy (Fig. 5C of reference 3). Expression of GFP-Pin1 had a profound effect on the morphology and organization of acinar formation. Colonies formed by GFP-Pin1-expressing cells exhibited the disorder in the nuclear polarity and cell arrangement without lumen inside, disruption of basement membrane and impairment in cell-cell junction (Fig.5B lower of reference 3). Furthermore, GFP-Pin1, but not GFP-expressing cells had cell surface spikes protruding into the Matrigel (arrows in Fig. 5D upper of reference 3). These results suggest that Pin1 overexpression can induce events associated with early stages of mammary tumorigenesis.

To verify these results in vivo we generated transgenic mice: An expression vector controlling Pin1 expression from the MMTV promoter was constructed, and injected blastocytes in our transgenic facility. We have now a number of transgenic Pin1 mice which we are currently screening for the expression of the Pin1 transgene. Subsequently, I will analyze the incidence of breast cancer in these mice.

In addition, we were able to obtain Pin1 knock-out mice from a Japanese group. I am currently performing breeding experiments with ras and neu transgenic mice. My question here is whether the Pin1 -/- background protects these mice from ras or neu induced breast cancers.The results from these experiments are still preliminary because I am still breeding and analyzing the mice. However, to date Pin1 +/- mice (heterozygous mice) that are transgenic for either the Her2neu, Ras or the c-myc oncogene show a significant delay in developing tumors (average and median 2 months).

GST-Pin1 proteins and its mutants were synthesized as indicated in task 2 and used for interaction studies (1-6).

In summary, the analysis of the data obtained in task 1 led to the unexpected discovery that Pin1 activates the transcription of cyclin D1 through binding and activating c-Jun and beta-catenin. Because cyclin D1 plays a much larger role in breast cancer carcinogenesis then nm23, the focus was shifted from studying the interaction on Pin1 with nm23 to its interaction with c-Jun and beta-catenin. The cell line experiments outlined in task 2 were performed but not with MCF-7 as originally planned. Instead non-transformed cells (MCF10A) were used which allowed us to show the transforming properties of Pin1 in cooperation with either Ras or the Her2/neu oncogene.

III. Key Research Accomplishments

- Examination of Pin1 levels in 50 primary breast cancer specimen
  Pin1 is overexpressed in 75% of breast cancers
- Correlation of Pin1 levels with clinicopathologic characteristics of the tumors
  Pin1 levels correlate with tumor grade and cyclin D1 levels
- Examination of the Pin1 interactions in vitro
  Pin1 is a transcriptional cotransactivator of the cyclin D1 promoter and interacts with c-jun as well as beta-catenin.
- Pin1's crucial role in Ras and Her2/neu signal transduction cascades were discovered

- Pin1-transgenic mice were generated Pin1 null mice obtained. These are currently being bred with various mouse breast cancer models

IV. Reportable Outcomes
- Manuscripts: see attachment

1. Kishi S, **Wulf G,** Nakamura M and Lu KP. Telomeric Protein Pin2/TRF1 Induces Mitotic Arrest and Apoptosis in Cells with Short Telomeres and is Down-regulated in Human Breast Tumors. **Oncogene** 2001, 20:1497-1508

2. **Wulf G,** Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. **EMBO J**. 2001 20: 3459-3472

3. Ryo A, Nakamura M*, **Wulf G***, Liou Y* and Lu KP. Prolyl isomerase Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. **Nature Cell Biology**, 2001 (3): 793-801
   *These authors contributed equally to this paper

4. Ryo A, Liou Y, **Wulf G**, Nakamura M, Lee S and Lu KP. PIN1 is an E2F Target Gene Essential for Neu/Ras-Induced Transformation of Mammary Epithelial Cells. **MCB** 2002, 22(15): 5281-5295

Patent
Zhou XZ, **Wulf G** and Lu KP. Pin1 as a Marker for Abnormal Cell Growth. U.S. Patent No.60/167,800

V. Conclusions
- The prolylisomerase Pin1 may promote tumor growth through the accumulation of cyclin D1 and through the potentiation Ras and Her2/neu oncogenic signalling. Because of its unique enzymatic function Pin1 could serve as a target for inhibitory drugs.

VI. References
1. **Wulf G,** Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. **EMBO J**. 2001 20: 3459-3472

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4. **Wulf** GM, Liou YC, Ryo A, Lee SW, Lu KP. Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. **J Biol Chem**. 2002 Oct 17 [epub ahead of print]

5. Kishi S, **Wulf G,** Nakamura M and Lu KP. Telomeric Protein Pin2/TRF1 Induces Mitotic Arrest and Apoptosis in Cells with Short Telomeres and is Down-regulated in Human Breast Tumors. **Oncogene** 2001, 20:1497-1508

6. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu K Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-nullphenotypes. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1335-40.

Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1

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Phosphorylation on serines or threonines preceding proline (Ser/Thr-Pro) is a major signaling mechanism. The conformation of a subset of phosphorylated Ser/Thr-Pro motifs is regulated by the prolyl isomerase Pin1. Inhibition of Pin1 induces apoptosis and may also contribute to neuronal death in Alzheimer's disease. However, little is known about the role of Pin1 in cancer or in modulating transcription factor activity. Here we report that Pin1 is strikingly overexpressed in human breast cancers, and that its levels correlate with cyclin D1 levels in tumors. Overexpression of Pin1 increases cellular cyclin D1 protein and activates its promoter. Furthermore, Pin1 binds c-Jun that is phosphorylated on Ser63/73-Pro motifs by activated JNK or oncogenic Ras. Moreover, Pin1 cooperates with either activated Ras or JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter. Thus, Pin1 is up-regulated in human tumors and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. Given the crucial roles of Ras signaling and cyclin D1 overexpression in oncogenesis, our results suggest that overexpression of Pin1 may promote tumor growth.
Keywords: cancer/c-Jun/cyclin D1/Pin1/Ras signaling

Introduction

The reversible phosphorylation of proteins on serine/threonine residues preceding proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of various cellular processes, including cell division and transcription (reviewed by Hunter and Karin, 1992; Nurse, 1994; Nigg, 1995; Treisman, 1996; Whitmarsh and Davis, 1996; Karin et al., 1997). For example, various growth factors and oncoproteins, such as oncogenic Ras, trigger a signaling cascade leading to the activation of c-Jun N-terminal kinases (JNKs), which phosphorylate c-Jun on Ser$^{63/73}$-Pro and enhance its transcriptional activity towards c-Jun target genes, including cyclin D1 (Binetruy et al., 1991; Smeal et al., 1991; Derijard et al., 1994; Hinds et al., 1994; Albanese et al., 1995, 1999; Fantl et al., 1995; Sicinski

et al., 1995; Robles et al., 1998; Bakiri et al., 2000). Overexpression of cyclin D1 often occurs in a variety of human cancers (Hunter and Pines, 1994), including ~50% of human breast tumors (Bartkova et al., 1994; Gillett et al., 1994; Lin et al., 2000). Importantly, cyclin D1 can act as an oncogene that contributes to cell transformation by complementing a defective E1A oncogene (Hinds et al., 1994). Conversely, inhibition of cyclin D1 expression causes growth arrest in tumor cells (Schrump et al., 1996; Arber et al., 1997; Driscoll et al., 1997; Kornmann et al., 1998). Moreover, knockout of cyclin D1 in mice blocks the proliferation of breast epithelial cells and retina, and inhibits tumor development in response to Ha-Ras (Fantl et al., 1995; Sicinski et al., 1995; Robles et al., 1998; Rodriguez-Puebla et al., 1999). These results indicate that cyclin D1 plays an important role during oncogenesis, acting as a downstream mediator of Ras activity during tumor development, and that phosphorylation of c-Jun on Ser$^{63/73}$-Pro motifs is an important mechanism for the Ras-dependent up-regulation of cyclin D1. However, it is not clear whether the c-Jun activity is further regulated after Pro-directed phosphorylation.

Compelling evidence supports an additional and crucial signaling mechanism, which affects the state of Pro-directed phosphorylation sites, namely the conformational change induced by phosphorylation-specific prolyl isomerization. Such conformational change can regulate protein function (Zhou et al., 1999). The phosphorylated Ser/Thr-Pro moiety exists in two distinct, slowly interconverting conformations: *cis* and *trans*. This conformational change introduces kinks into a peptide chain, thereby determining protein structure and function (Fischer, 1994; Galat and Metcalfe, 1995; Schmid, 1995; Hunter, 1998; Zhou et al., 1999). Significantly, phosphorylation on Ser/Thr-Pro motifs further restrains the already slow *cis/trans* prolyl isomerization of peptide bonds (Yaffe et al., 1997; Schutkowski et al., 1998), and also renders them resistant to the catalytic action of conventional peptidyl-prolyl *cis/trans* isomerases (PPIases), including cyclophilins and FK506-binding proteins (Yaffe et al., 1997). In contrast, Pin1 represents a new subfamily of highly conserved and phosphorylation-specific PPIases that isomerize only the phosphorylated Ser/Thr-Pro bonds, and not their non-phosphorylated counterparts (Yaffe et al., 1997). Pin1 contains an N-terminal WW domain and a C-terminal PPIase domain (Lu et al., 1996; Ranganathan et al., 1997). The WW domain functions as a pSer/Thr-binding module, interacting with specific pSer/Thr-Pro motifs present in a defined subset of phosphoprotein substrates, including Cdc25C, tau, Myt1, S6 kinase, Rab4 and the C-terminal domain of RNA polymerase II (Lu et al., 1999b). At the substrate, the PPIase domain of Pin1 isomerizes specific pSer/Thr-Pro bonds, and regulates protein function and dephosphoryl-
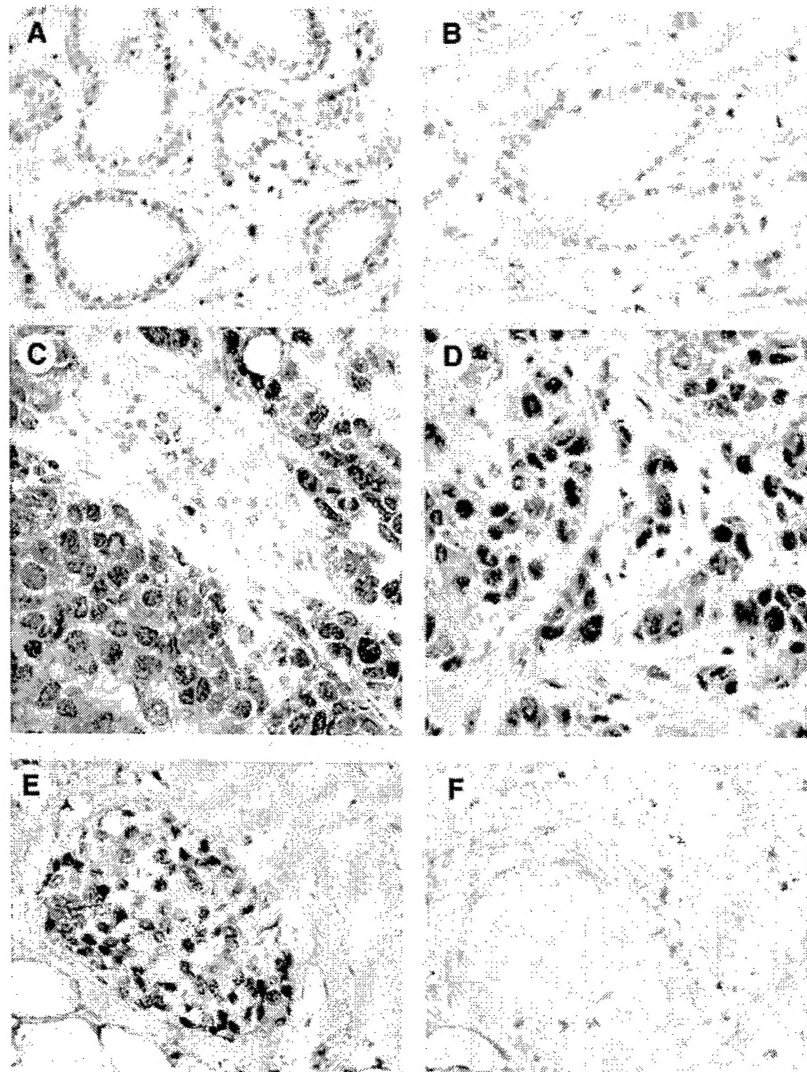
Fig. 1. Immunostaining of Pin1 in human breast cancer. Sections from paraffin-embedded tissues were subjected to an antigen retrieval treatment, followed by immunostaining with anti-Pin1 antibodies. Non-cancerous tissues (**A** and **B**; normal breast with mild fibrocystic changes) show weak, but detectable, Pin1 staining, while invasive ductal carcinomas (**C** and **D**) or ductal carcinoma *in situ* (**E**) show intense Pin1 staining. To show the specificity of Pin1 antibodies, Pin1-specific antibodies were first depleted using GST–Pin1 beads and then used to stain the breast sections (**F**).

ation (Yaffe *et al.*, 1997; Shen *et al.*, 1998; Lu *et al.*, 1999b; Zhou *et al.*, 1999, 2000). For example, in the case of Cdc25C, Pin1 binds phosphorylated Cdc25C, and inhibits its activity to dephosphorylate and activate Cdc2 (Shen *et al.*, 1998; Zhou *et al.*, 2000). However, in the case of tau, Pin1 binds Alzheimer's disease-associated phosphorylated tau and restores its biological function to promote microtubule assembly (Lu *et al.*, 1999a; Zhou *et al.*, 2000). These results indicate that Pin1 plays an important role in the regulation of a defined subset of phosphorylated proteins.

Functionally, Pin1 is critical for cell proliferation *in vivo*. Temperature-sensitive mutations or deletion of the Ess1 gene (the Pin1 homologue in budding yeast) result in mitotic arrest and nuclear fragmentation (Hanes *et al.*, 1989; Hani *et al.*, 1995, 1999; Lu *et al.*, 1996). These

arrested cells have defective 3′ end formation of premRNA, and decreased levels of some mRNAs (Hani *et al.*, 1999; Wu *et al.*, 2000). However, it remains to be determined whether these defects are primarily due to the effect of Ess1 on the general transcription machinery, as suggested, or secondarily due to the fact that these cells are arrested in mitosis with fragmented nuclei, or both. Inhibition of the Pin1 function in human tumor cells using expression of the Pin1 antisense RNA or dominant-negative mutants induces mitotic arrest and apoptosis (Lu *et al.*, 1996; Rippmann *et al.*, 2000; P.J.Lu, X.Z.Zhou, Y.-C.Liou, J.P.Noel and K.P.Lu, submitted). Similarly, depletion of Pin1 in Alzheimer's disease brain may also contribute to neuronal death (Lu *et al.*, 1999a). Furthermore, depletion of Pin1 in *Xenopus* extracts induces premature mitotic entry and disrupts a DNA

replication checkpoint (Winkler *et al.*, 2000). These results together suggest that the level and function of Pin1 are pivotal for cell proliferation. However, the level and role of Pin1 in human cancer have not yet been described.

Here we show that Pin1 is overexpressed in most human breast cancer cell lines and many human breast cancer tissues. Furthermore, the Pin1 levels correlate significantly with the grade of the tumors, according to Bloom and Richardson's classification system (Bloom and Richardson, 1957), and with the level of cyclin D1 in the tumors. Moreover, Pin1 increases levels of cellular cyclin D1 mRNA and protein, and activates its promoter through the AP-1 site. Importantly, Pin1 binds to phosphorylated c-Jun and increases its transcriptional activity towards the cyclin D1 promoter, in cooperation either with activated JNK or oncogenic Ras. The effects of Pin1 on the c-Jun transcriptional activity depend on both the isomerase activity of Pin1 and phosphorylation of c-Jun on Ser$^{63/73}$. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun. These results demonstrate that Pin1 is up-regulated in human tumor samples and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. Given the crucial roles of the activated Ras signaling and cyclin D1 overexpression in the development of cancer, our results suggest that overexpression of Pin1 may promote tumor growth.

Results

Pin1 is overexpressed in human breast tumors and its levels correlate with the tumor grade

To examine the role of Pin1 in cancer, we examined the expression of Pin1 in normal human breast tissues and breast tumors by immunohistochemistry and immunoblotting with affinity-purified anti-Pin1 antibodies, as described earlier (Lu *et al.*, 1999a). Normal breast epithelial cells showed weak but detectable Pin1 staining primarily in the nucleus (Figure 1A and B). In contrast, carcinoma cells were strongly positive for the Pin1 staining (Figure 1C–E), while surrounding normal connective tissue, blood vessels, adipose and stromal cells stained only weakly for Pin1 (Figure 1E). In these tumor cells, Pin1 staining was detected at high levels in the cytoplasm, in addition to intensive staining in the nucleus (Figure 1C–E). To ensure that these signals indeed represent Pin1, the Pin1-specific antibodies were depleted using glutathione *S*-transferase (GST)–Pin1 beads prior to immunostaining. Figure 1F shows that the Pin1-depleted antibodies showed no immunoreactivity, confirming the specificity of the antibodies, as described (Lu *et al.*, 1999a). Immunohistochemistry in other cancer types revealed high Pin1 levels in some tumors, including colon cancer, lymphomas, melanoma, prostate and brain tumors, but rarely in others, such as sarcoma (data not shown). Since we had access to a large number of breast cancer samples, we focused this study on breast cancer.

To evaluate Pin1 expression in breast cancer quantitatively, we ground fresh, normal or tumor breast tissues in liquid nitrogen and subjected the lysates directly to immunoblotting analysis with various antibodies, followed by semi-quantification of protein levels using Imagequant, as described (Lu *et al.*,

1999a). Pin1 was generally detected as a doublet in immunoblots, especially in tumor tissues where Pin1 was overexpressed. Upon dephosphorylation with protein phosphatases PP2A and PP1, or calf intestine phosphatase (CIP), the intensity of the upper band decreased, while the lower one increased (Figure 2B). In addition, Pin1 displays a mitosis- and phosphorylation-specific mobility shift during the cell cycle (P.J.Lu, X.Z.Zhou, Y.-C.Liou, J.P.Noel and K.P.Lu, submitted). These results indicate that the Pin1 doublet is likely to be due to the electrophoretic mobility difference of phosphorylated and dephosphorylated Pin1. Interestingly, the upper phosphorylated band appeared to be predominant in the normal tissues, whereas the lower dephosphorylated band was more abundant in the cancerous tissues where Pin1 was overexpressed (Figure 2A), suggesting that there are more mitotic cells and/or the kinase(s) responsible for the Pin1 phosphorylation might be limited in tumor cells.

To compare the levels of Pin1 in different human tissues, we used actin as an internal control, and expressed the Pin1 level in each sample as a Pin1:actin ratio. We defined Pin1 overexpression as higher than the mean plus three times the standard deviation ($\bar{x}$ + 3 SD) of normal controls (Figure 2C; Table I). In 10 normal and 51 primary human breast cancer tissues examined, we observed striking differences in the levels of Pin1 protein expression (Figure 2A and C). One out of four DCIS tumors, 20 out of 28 (71.4%) grade II tumors and 17 out of 19 (89.5%) grade III tumors, according to Bloom and Richardson's classification system, overexpressed Pin1 (Figure 2C). Although we observed considerable inter-individual variations, especially in grade II and III tumors (Figure 2C), the mean expression level of Pin1 was ~10 times higher in cancer samples than in the normal controls (Table I). Furthermore, Pin1 levels positively correlated with the Bloom and Richardson grade in invasive breast cancer, as analyzed by the Kruskal–Wallis test (Glantz, 1997) (Figure 2B; Table II). Similar results were also obtained using a monoclonal antibody against Pin1 for immunostaining and immunoblotting analyses (data not shown). The levels of Pin1 in four cell lines derived from human breast cancers were considerably higher than those in either normal mammary epithelial cells or two cell lines established from normal mammary epithelial cells (Figure 2D). Together, these results indicate that Pin1 is overexpressed in many human breast cancer tissues and cell lines, and its levels are correlated with the tumor grade.

Up-regulation of Pin1 correlates with cyclin D1 levels in breast tumor tissues and elevates cellular cyclin D1 expression in breast cell lines

Amongst other breast cancer tumor markers, Pin1 levels did not appear to correlate with either estrogen receptor or HER2/neu expression, but did correlate significantly with cyclin D1 overexpression (Tables I and II). As shown previously (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), cyclin D1 was overexpressed in ~50% of the patient samples (24 out of 51 cases). Importantly, Pin1 was overexpressed in 20 out of 24 cyclin D1-overexpressing tumors, and Pin1 levels in cyclin D1-overexpressing
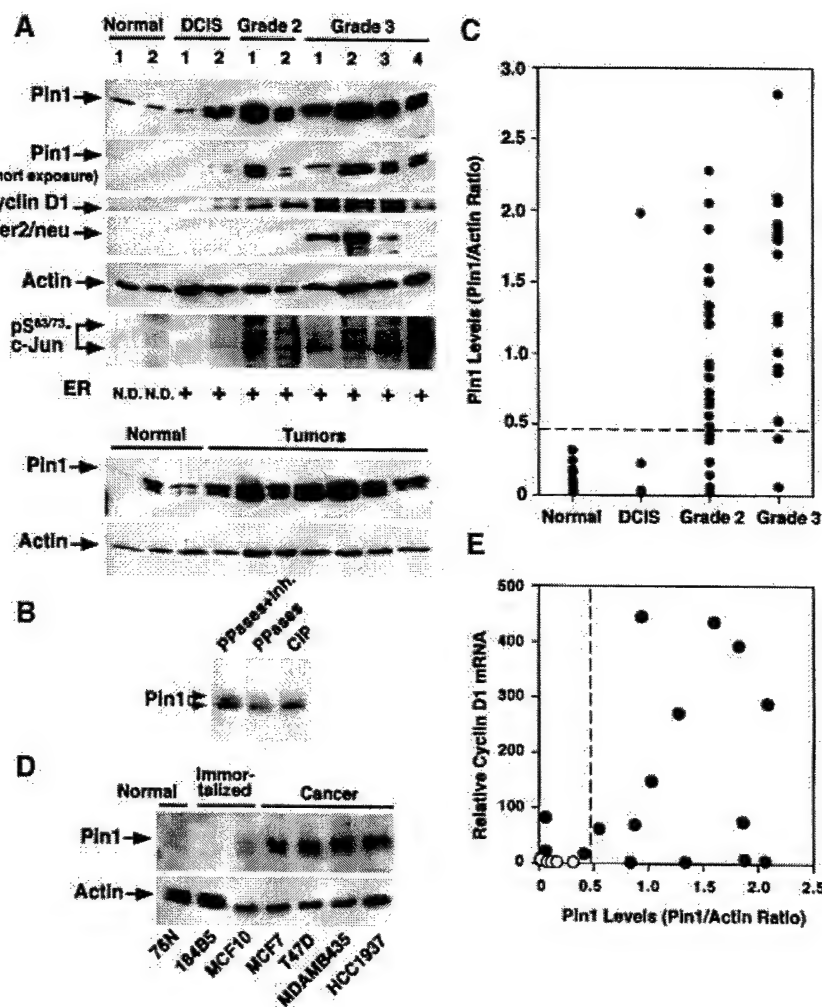
Fig. 2. Pin1 overexpression in human breast cancer cell lines and patient tissues, and its correlation with the Bloom and Richardson grade of tumors. (**A**) Comparison of Pin1 levels and known breast tumor markers in normal and cancerous human breast tissues. Normal breast and cancer tissues were pulverized in liquid nitrogen, and equal amounts of total protein were separated on SDS-containing gels and transferred to membranes. The membranes were cut into five pieces and subjected to immunoblotting analysis using antibodies to Pin1, cyclin D1, HER2/neu, phosphorylated Ser$^{63/73}$-c-Jun and actin, respectively. The estrogen receptor status was determined by radioimmunoassay and defined as positive when its levels were >10 fmol/l. The estrogen receptor status in normal controls was not determined (N.D.). Note that Pin1 was detected in immunoblots as a doublet due to phosphorylation. (**B**) Phosphatase treatment abolishes the double-band pattern of Pin1 in immunoblots. Tumor cell lysates were treated either with a mixture of PP1 and PP2A (PPases) in the presence (lane 1) or absence (lane 2) of the phosphatase inhibitor okadaic acid (Inh.), or CIP (lane 3). (**C**) Pin1 levels in 10 normal breast tissues and different stages of 51 human breast cancer samples. Pin1 levels were determined by immunoblotting analysis, as in (A), and semi-quantified using Imagequant. Actin was used as an internal control, and the Pin1 level in each sample was expressed as the Pin1:actin ratio. (**D**) Comparison of Pin1 levels in mammary epithelial cell lines. The same amounts of total lysates prepared from normal human mammary epithelial cell lines (Normal), spontaneously immortalized normal human mammary epithelial cell lines (Immortalized) and human breast carcinoma-derived cell lines (Cancer) were subjected to immunoblotting analysis with Pin1 or actin antibodies. (**E**) Correlation of Pin1 protein levels with cyclin D1 mRNA. RNA was isolated from six normal and 16 cancerous tissues, cDNA synthesized and subjected to real-time PCR for the quantitative analysis of cyclin D1 mRNA expression. The Pearson correlation coefficient was 0.47 (p <0.05).

tumors were on average about twice as high as those in cyclin D1-negative tumors (Figure 2A; Table II). In order to establish a link between Pin1 overexpression and cyclin D1 transcription, we performed quantitative real-time PCR to detect cyclin D1 mRNA expression in 6 out of the 10 normal tissues and 16 of the 51 breast cancer tissues, from which we were able to isolate total RNA. Figure 2E shows relative cyclin D1 mRNA levels as a function of Pin1 protein levels. While a few patients had

high Pin1 but low cyclin D1 mRNA levels, all but one patient with high cyclin D1 mRNA levels also displayed high Pin1 levels, which is consistent with the results on cyclin D1 protein levels (Table II). Statistical analysis revealed that there was again a positive correlation between Pin1 protein levels and cyclin D1 mRNA expression ($r = 0.47$, p <0.05).

The correlation between Pin1 and cyclin D1 expression suggested that overexpression of Pin1 might increase the

Table I. Clinical and pathological characteristics of breast tissues

	Normal	Carcinoma				
		Total	*In situ*	Grade 2	Grade 3	
Pin1 positive	0/10[a]	38/51 (75%)	1/4 (25%)	20/28 (71%)	17/19 (89%)	
$\bar{x} \pm$ SD	0.114 ± 0.106	1.072 ± 0.719	0.564 ± 0.948	0.924 ± 0.609	1.399 ± 0.717	
Cyclin D1	0/10	24/51 (47%)	2/4 (50%)	10/28 (36%)	12/19 (63%)	
HER2/neu	0/10	8/51 (16%)	0/4 (0%)	4/28 (14%)	4/19 (21%)	
Estrogen receptor	N.D.[b]	34/50[c] (68%)	3/4 (75%)	20/28 (71%)	11/18 (61%)	
Age median (range)	57 (22–91)	65 (28–90)	72 (43–80)	65 (31–90)	60 (28–78)	

Tumors were pathologically classified into ductal carcinoma *in situ* (*in situ*) and invasive grade 2 and 3 carcinoma, according to the criteria of Bloom and Richardson. Levels of Pin1 in tissues were determined by immunoblotting analysis and semi-quantified using Imagequant, with the results being expressed as Pin1/actin ratio. Pin1 was defined positive when the Pin1/actin ratio was higher than the mean plus three times the standard deviation ($\bar{x} \pm$ 3 SD) of normal controls. Cyclin D1 and HER2/neu were determined by immunoblotting and categorized as either positive or negative by the presence or absence of the respective proteins. Estrogen receptor was defined positive when its levels were >10 fmol/l, as determined by radioimmunoassay.
[a]Number of cases examined.
[b]Estrogen receptors in controls not determined.
[c]Estrogen receptor determination on one patient not available.

Table II. Correlation of the Pin1 level with clinical and pathological characteristics

	No. of cases	Pin1 level ($\bar{x} \pm$ SD)	p
Normal	10	0.114 ± 0.106	<0.0001[b]
Tumor	51	1.072 ± 0.716	
Tumor grade			
grade 2	28	0.924 ± 0.609	0.02[b]
grade 3	19	1.399 ± 0.717	
Cyclin D1[a]			
positive	24	1.364 ± 0.715	0.01[b]
negative	27	0.824 ± 0.631	
HER2/neu[a]			
positive	8	1.317 ± 0.732	0.10
negative	43	1.027 ± 0.713	
Estrogen receptor[a]			
positive	34	1.011 ± 0.718	0.32
negative	16	1.238 ± 0.720	

The significance of the differences in Pin1 levels between various clinical and pathological categories was analyzed by the Kruskal–Wallis test.
[a]Analyses performed only on tumors.
[b]The differences are statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$.

expression of endogenous cyclin D1. To examine this possibility, we transiently transfected a Pin1 expression construct into two breast cancer-derived cell lines, MCF7 and T47D cells, and then examined the effects on endogenous cyclin D1 levels. Pin1 overexpression led to 2- to 3-fold increases in cyclin D1 protein levels in both cell lines, while the expression of actin remained constant (Figure 3A). To examine whether the depletion of Pin1 affected cyclin D1 expression, we used MCF7 and HeLa cells because their Pin1 levels can be increased or decreased by expressing a sense or antisense Pin1 construct, respectively (Figure 3B), as described previously (Lu *et al.*, 1996). Overexpression of Pin1 significantly increased the levels of cyclin D1 protein and mRNA in both cells (Figure 3B and C and data not shown). In contrast, depletion of Pin1 significantly reduced the levels of cyclin D1 protein and mRNA in MCF7 cells (Figure 3B and C). Since these experiments were performed between

24 and 36 h after transfection, and since manipulation of Pin1 levels affects the cell cycle only after 48–72 h post-transfection (Lu *et al.*, 1996), the observed effects of Pin1 on cyclin D1 are unlikely to be related to cell cycle arrest. These results indicate that high levels of Pin1 correlate with the overexpression of cyclin D1 on both RNA and protein levels in human breast cancer tissues, and that overexpression of Pin1 increases cellular cyclin D1 mRNA and protein levels in cell lines.

Pin1 activates the cyclin D1 promoter
Although cyclin D1 overexpression is found in ~50% of breast cancer patients (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), gene amplification accounts for only 10% of these cases (Fantl *et al.*, 1993). Therefore, other mechanisms, such as up-regulation of gene transcription, must play a substantial role in the overexpression of cyclin D1. To determine whether Pin1 regulates the transcription of cyclin D1, we measured the effects of Pin1 on the cyclin D1 promoter using cyclin D1–luciferase reporter constructs. Two cyclin D1–reporter constructs were tested: one (–1745CD1) corresponds to the original fragment of cyclin D1 5' sequence cloned from the PRAD1 breakpoint (Motokura and Arnold, 1993), and the other (–964CD1) is the minimum 5' sequence that retains the responsiveness to activated Ras (Albanese *et al.*, 1995). Both –1745CD1 and –964CD1 reporters were strongly activated in response to expression of Pin1 both in MCF7 and HeLa cells (Figure 3D and E). These results indicate that Pin1 activates the cyclin D1 promoter and that the –964CD1 promoter fragment retains the complete responsiveness to Pin1.

It has recently been shown that Pin1/Ess1p binds the phosphorylated C-terminal domain of RNA polymerase II and may regulate the general transcription machinery in yeast (Wu *et al.*, 2000). To determine whether activation of the cyclin D1 promoter by Pin1 is due to its effect on the general transcription machinery, we examined the effect of Pin1 on several other unrelated promoters. To detect the maximal effect of Pin1 on various promoters, we used 500 ng of Pin1 cDNA per transfection. In contrast, out of many other promoters examined, including thymidine

kinase (TK), c-fms (M-CSF receptor) and MMTV promoters, Pin1 either had no effect or had minor transactivating effects (Figure 3D and E), indicating that activation of the general transcription machinery by Pin1 is very low, which is consistent with a recent report (Chao *et al.*, 2001). Therefore, the above results indicate that Pin1 specifically activates the cyclin D1 promoter.

To further confirm the specificity of the Pin1 action on the cyclin D1 promoter, we identified the element in the cyclin D1 promoter that is responsible for Pin1 activation.

The –964CD1 promoter fragment contains binding sites for various transcription factors, including a CREB site, four TCF sites, three Ets sites and one AP-1 site (Albanese *et al.*, 1995; Tetsu and McCormick, 1999) (Figure 3F). To determine which promoter is necessary for Pin1 responsiveness, we used two deletion constructs containing either 22 bp (–22CD1) or 163 bp (–163CD1) of the cyclin D1 promoter as reporters. Low concentrations (50–200 ng) of Pin1 did not have any significant transactivating effect either on the –22CD1 or the –163CD1 reporter (Figure 3F
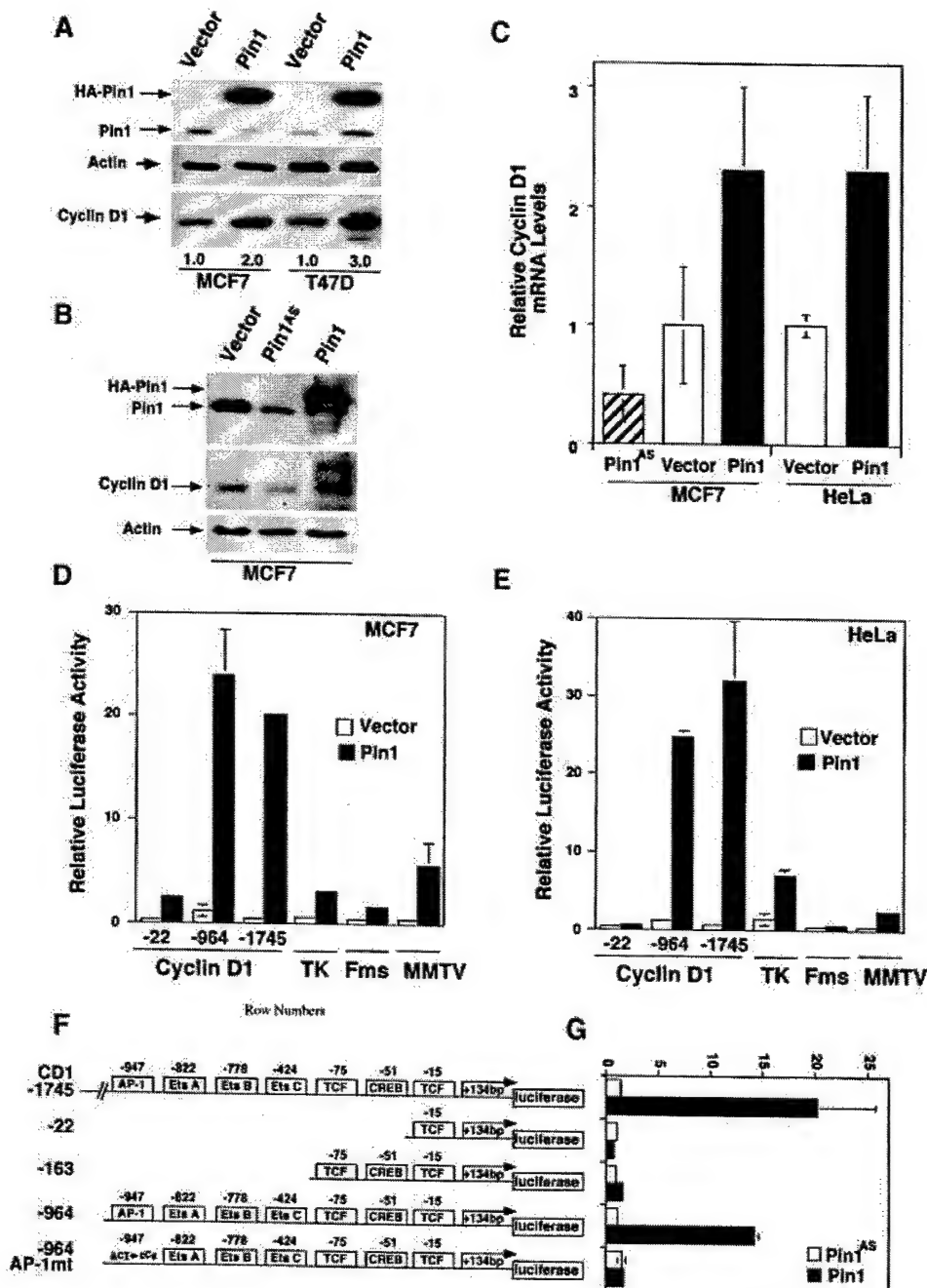
and G), while at high concentrations (>200 ng per transfection) Pin1 could also transactivate the −163CD1 promoter containing the TCF sites (Ryo *et al.*, 2001). At low concentrations, i.e. ≤200 ng, Pin1 significantly transactivated both the −1745CD1 and −964CD1 promoters (Figure 3F and G). These results confirm that Pin1 does not affect the cyclin D1 promoter activity via the general transcriptional machinery but through specific sequences such as the AP-1 and/or Ets sites. To examine the importance of the AP-1 site, we used a mutant promoter, −964CD1AP-1mt, containing only two base-pair substitutions at the consensus AP-1 site, as described (Albanese *et al.*, 1995). Elimination of the AP-1 site almost completely abolished the ability of Pin1 to activate the cyclin D1 promoter (Figure 3F and G). These results indicate that the AP-1 site is essential for activation of the cyclin D1 promoter by Pin1.

Pin1 binds c-Jun phosphorylated on Ser63/73-Pro motifs

The AP-1 site mutation in the cyclin D1 promoter that disrupts the Pin1 transactivating activity also abolishes cyclin D1 expression induced by the activation of Ras or c-Jun (Albanese *et al.*, 1995), suggesting that Pin1 might affect the same pathway as that regulated by Ras or c-Jun. Activation of Ras triggers a signaling cascade, leading to activation of the c-Jun N-terminal kinase JNK, which phosphorylates c-Jun on Ser$^{63/73}$-Pro to increase its transcriptional activity towards its target genes, including cyclin D1 (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Derijard *et al.*, 1994; Hinds *et al.*, 1994; Albanese *et al.*, 1995, 1999; Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Bakiri *et al.*, 2000). In fact, Ras-mediated tumorigenesis depends on signaling pathways with cyclin D1 as an important intermediary protein (Robles *et al.*, 1998). Since Pin1 binds and regulates the function of a defined subset of proteins phosphorylated on certain Ser/Thr-Pro motifs (Shen *et al.*, 1998; Lu *et al.*, 1999a), it is possible that Pin1 might activate the cyclin D1 promoter via modulation of the the activity of phosphorylated c-Jun.

A well established and successful procedure to identify Pin1 substrates has been the use of GST–Pin1 pulldown experiments to determine whether Pin1 binds to c-Jun, and whether the binding depends on phosphorylation of c-Jun on specific Ser-Pro motifs, as demonstrated for many other Pin1 substrates (Yaffe *et al.*, 1997; Crenshaw *et al.*, 1998; Shen *et al.*, 1998; Lu *et al.*, 1999b). To increase phosphorylation of c-Jun on Ser$^{63/73}$-Pro, we co-transfected c-Jun with a constitutively activated form of JNK (Derijard *et al.*, 1994). Alternatively, we co-transfected c-Jun with a further upstream activator, the oncogenic Harvey-Ras (Ha-Ras or RasL61), which activates a MAK kinase pathway, leading to activation of JNK (Smeal *et al.*, 1991; Derijard *et al.*, 1994). To reduce phosphorylation of c-Jun on Ser$^{63/73}$-Pro, we co-transfected c-Jun with the dominant-negative Ras (DN-Ras or RasN17) (Smeal *et al.*, 1991; Derijard *et al.*, 1994). As expected, phosphorylation of c-Jun on Ser$^{63/73}$-Pro was increased to similar extents by either activated JNK or Ha-Ras, but significantly decreased by DN-Ras, as detected by antibodies specifically recognizing phosphorylated Ser$^{63/73}$ in c-Jun (Figure 4A and B). Notably, following activation of JNKs by UV radiation or serum stimulation, c-Jun has been shown to be phosphorylated on several Ser-Thr sites, which resulted in a considerable shift in electrophoretic mobility of the protein, migrating as multiple bands in SDS gels (Ui *et al.*, 1998). Furthermore, mutation of c-Jun on Ser63 and Ser73 abolishes the mobility shift (Ui *et al.*, 1998). We observed a similar mobility shift for wild-type c-Jun, but not c-Jun$^{S63/73A}$, after co-transfection either with Ha-Ras or activated JNK (Figure 4A–D). Importantly, although there was no binding between GST and c-Jun, weak binding between GST–Pin1 and c-Jun was detected when only c-Jun was transfected (Figure 4C). Furthermore, the binding was significantly increased by co-transfection either with activated JNK or oncogenic Ha-Ras, but not with DN-Ras (Figure 4C). Moreover, c-Jun bound by Pin1 was also phosphorylated on Ser$^{63/73}$-Pro, as indicated by phosphorylated Ser$^{63/73}$-specific antibodies (Figure 4D). To examine further the importance of phosphorylation on Ser$^{63/73}$ for Pin1 binding, we used a c-Jun mutant, c-Jun$^{S63/73A}$, which contains double Ala substitutions at Ser63 and Ser73 (Smeal *et al.*, 1991). In contrast to wild-type c-Jun, the mutant protein did not display a significant mobility shift and was not recognized by phosphorylated Ser$^{63/73}$-specific antibodies (Figure 4A and B), as shown previously (Ui *et al.*, 1998).

Fig. 3. Pin1 elevates cyclin D1 protein and activates the cyclin D1 promoter via the AP-1 site. (A) Increase in cellular cyclin D1 protein by Pin1. MCF7 or T47D cells were transfected with Pin1 or control vector, followed by immunoblotting analysis of the cell lysates with antibodies against Pin1 and cyclin D1, with actin as a control. Cyclin D1 levels were semi-quantified using Imagequant and are presented below the image; the level in the vector control was defined as 1. (B) Manipulation of Pin1 levels in cells causes changes in cyclin D1 levels. MCF7 cells were transiently transfected with the control vector or a construct expressing HA-Pin1 or antisense Pin1 (Pin1^{AS}), followed by immunoblotting analysis with anti-cyclin D1, -Pin1 or -actin antibodies. (C) Overexpression and depletion of Pin1 increase and decrease levels of cyclin D1 mRNA. MCF7 or HeLa cells were transfected with constructs encoding for Pin1 sense, antisense or vector control as indicated in the figure. After 24 h, mRNA was isolated, cDNA synthesized and subjected to real-time PCR to obtain relative cyclin D1 mRNA levels. (D and E) Activation of cyclin D1, but not TK, c-fms or MMTV promoter by Pin1. MCF7 (D) or HeLa (E) cells were transiently transfected with Pin1 or the vector and various reporter constructs, followed by assaying the luciferase activity. pRL-TK *Renilla* luciferase reporter construct was co-transfected in each sample to normalize for transfection efficiency. The activity of the reporter luciferase was expressed relative to that in control vector-transfected cells, which is defined as 1. All results are expressed as $\bar{x}$ ± SD of independent duplicate cultures. Note that to detect the maximal effect of Pin1 on various promoters, we used 0.5 μg of Pin1 cDNA per transfection in this experiment, which was higher than in other experiments described here. (F) Schematic representation of cyclin D1 (CD1) pA3LUC basic reporter constructs and its mutants. Possible transcription factor-binding sites are indicated. −964CD1AP-1mt was same as the wild-type −964CD1construct except for two base-pair substitutions at the consensus AP-1 site. (G) Activation of the cyclin D1 promoter by Pin1 via the AP-1 site. HeLa cells were co-transfected with various cyclin D1 reporter constructs as indicated in (F) and Pin1 sense or antisense (Pin1^{AS}) construct, followed by assaying the luciferase activity. Note that for this experiment 200 ng Pin1 sense or antisense cDNA were used, while in subsequent co-transfection experiments only 50 ng/assay were used.
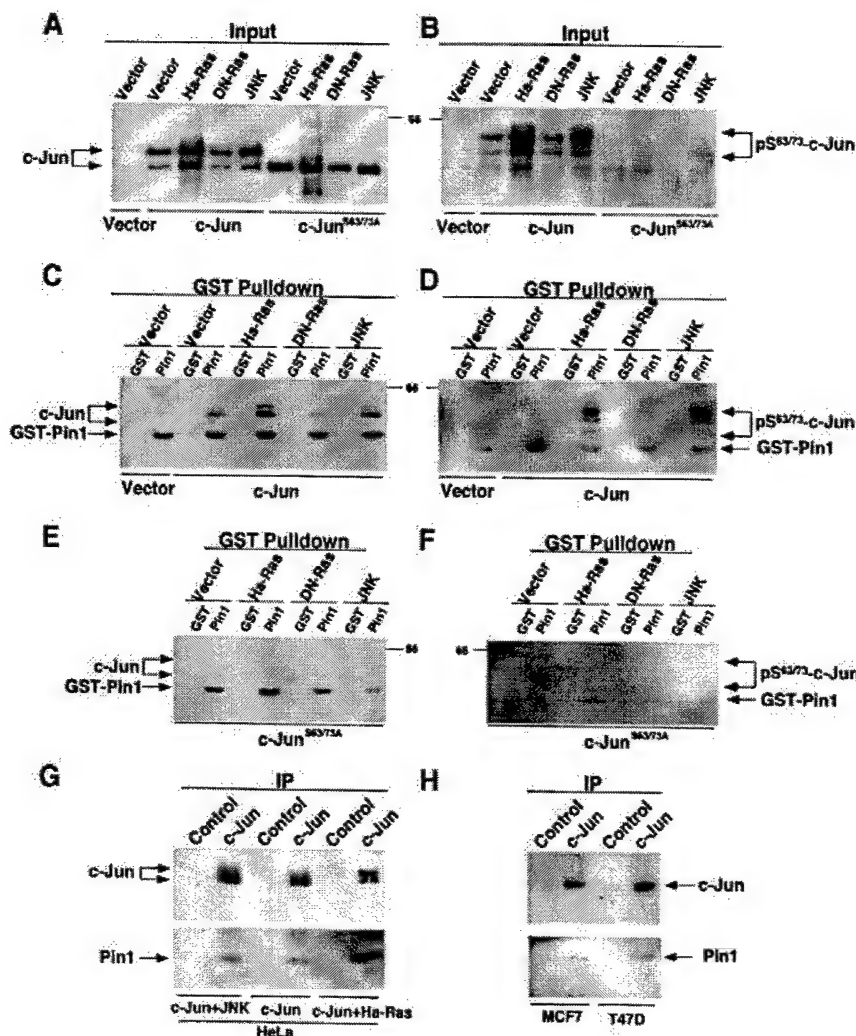
Fig. 4. Pin1 binds to c-Jun phosphorylated on $Ser^{63/73}$-Pro. (**A** and **B**) Modulation of c-Jun phosphorylation by Ras or JNK. HeLa cells were co-transfected with c-Jun or c-Jun$^{S63/73A}$ and Ha-Ras, DN-Ras, activated JNK or control vector. Cells were harvested and cellular proteins were subjected to immunoblotting analysis with antibodies against c-Jun (A) or phosphorylated $Ser^{63/73}$-c-Jun (B). (**C** and **D**) Interaction between Pin1 and c-Jun phosphorylated on $Ser^{63/73}$-Pro. The same cellular proteins as those described in (A) were incubated with GST–agarose beads that had been pre-incubated with either GST alone or GST–Pin1. Proteins associated with the beads were subjected to immunoblotting analysis with antibodies against c-Jun (C) or phosphorylated $Ser^{63/73}$-c-Jun (D). Note that GST–Pin1 was non-specifically recognized by monoclonal antibodies, as shown previously (Yaffe *et al.*, 1997; Lu *et al.*, 1999b). (**E** and **F**) No interaction between Pin1 and c-Jun$^{S63/73A}$. The same cellular proteins as those described in the (A) were incubated with GST–agarose beads containing GST or GST–Pin1, and bound proteins were subjected to immunoblotting analysis with antibodies against c-Jun (E) or phosphorylated $Ser^{63/73}$-c-Jun (F). (**G** and **H**) Co-immunoprecipitation of transfected (G) or endogenous (H) c-Jun with endogenous Pin1. HeLa cells were co-transfected with c-Jun and Ha-Ras or JNK. c-Jun was immunoprecipitated from transfected HeLa cells (G) or non-transfected breast cancer cell lines (H) with polyclonal c-Jun antibodies or non-related antibodies (Control), and then subjected to immunoblotting using monoclonal anti-c-Jun antibodies (upper panel) or anti-Pin1 antibodies (lower panel).

Importantly, little, if any, mutant protein was precipitated by Pin1 (Figure 4E and F). These results indicate that phosphorylation of c-Jun on $Ser^{63/73}$-Pro is important for the Pin1 binding. Thus, Pin1 binds to c-Jun via phosphorylated $Ser^{63/73}$-Pro motifs.

To confirm these GST–Pin1 protein pulldown results, we performed co-immunoprecipitation experiments between endogenous Pin1 and transfected c-Jun in the presence or absence of activated JNK or Ha-Ras, as well as co-immunoprecipitations between endogenous Pin1 and c-Jun in breast cancer cell lines expressing high levels of

both proteins. Endogenous Pin1 was detected in anti-c-Jun immunoprecipitates from transfected (Figure 4G) and non-transfected cells (Figure 4H). Furthermore, more Pin1 was co-immunoprecipitated by anti-c-Jun antibodies if c-Jun was co-transfected with activated JNK or Ha-Ras (Figure 4G). These results indicate that Pin1 binds c-Jun *in vivo* in breast cancer cell lines, and that the binding is increased when c-Jun is phosphorylated on $Ser^{63/73}$-Pro motifs by activated JNK or Ha-Ras. These results demonstrate that Pin1 binds phosphorylated c-Jun both *in vitro* and *in vivo*.
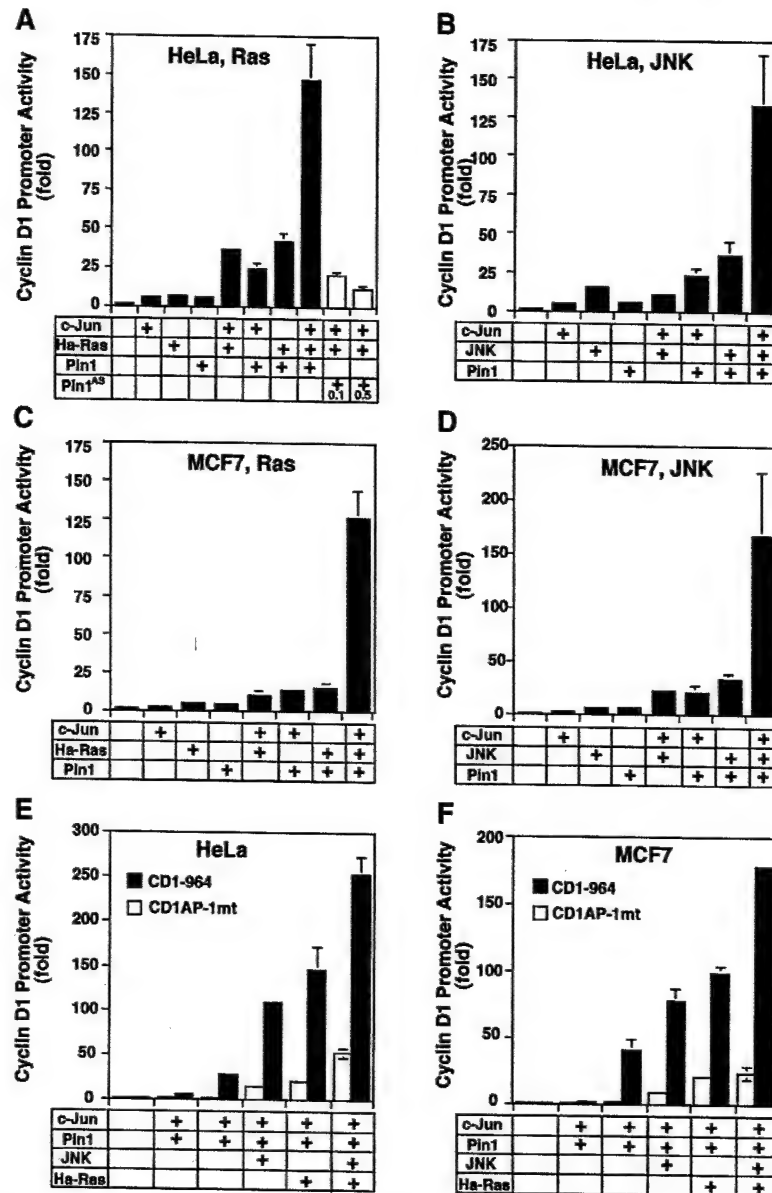
Fig. 5. Pin1 cooperates either with Ha-Ras or activated JNK in enhancing the activity of c-Jun to activate the cyclin D1 promoter. HeLa cells (A, B and E) or MCF7 (C, D and F) were co-transfected with vector, c-Jun, or c-Jun with or without Ha-Ras (A and C) or activated JNK (B and D) and then subjected to the luciferase assay with the –964 cyclin D1 Luc promoter construct as reporter gene. In the same system, a reporter gene construct with an AP-1 site mutant fails to respond to Pin1 in combination with c-Jun, JNK or Ha-Ras (E and F).

Pin1 cooperates with either oncogenic Ha-Ras or activated JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter

Given that Pin1 binds phosphorylated c-Jun, we asked whether Pin1 also modulates the activity of c-Jun. To address this question, we examined the effect of Pin1 on the transcriptional activity of c-Jun towards the cyclin D1 promoter in the presence or absence of Ha-Ras or activated JNK. When Pin1 cDNA was co-transfected with c-Jun, Pin1 cooperated moderately with c-Jun in activating the cyclin D1 promoter in both MCF7 and HeLa cells

(Figure 5). The activity of the cyclin D1 promoter in cells co-transfected with Pin1 and c-Jun was 3- to 5-fold higher than that in cells transfected with either Pin1 or c-Jun alone (Figure 5A–D). The most dramatic potentiation of cyclin D1 reporter gene activity was observed when c-Jun was activated by JNK or Ha-Ras in the presence of Pin1; cyclin D1 promoter activity was increased up to 150-fold, or higher, in both cell lines (Figure 5A–D). The combination of JNK, Ras, c-Jun and Pin1 resulted in a further small increase in transactivation (Figure 5E and F, last bars), consistent with the idea that Ras and JNK act on
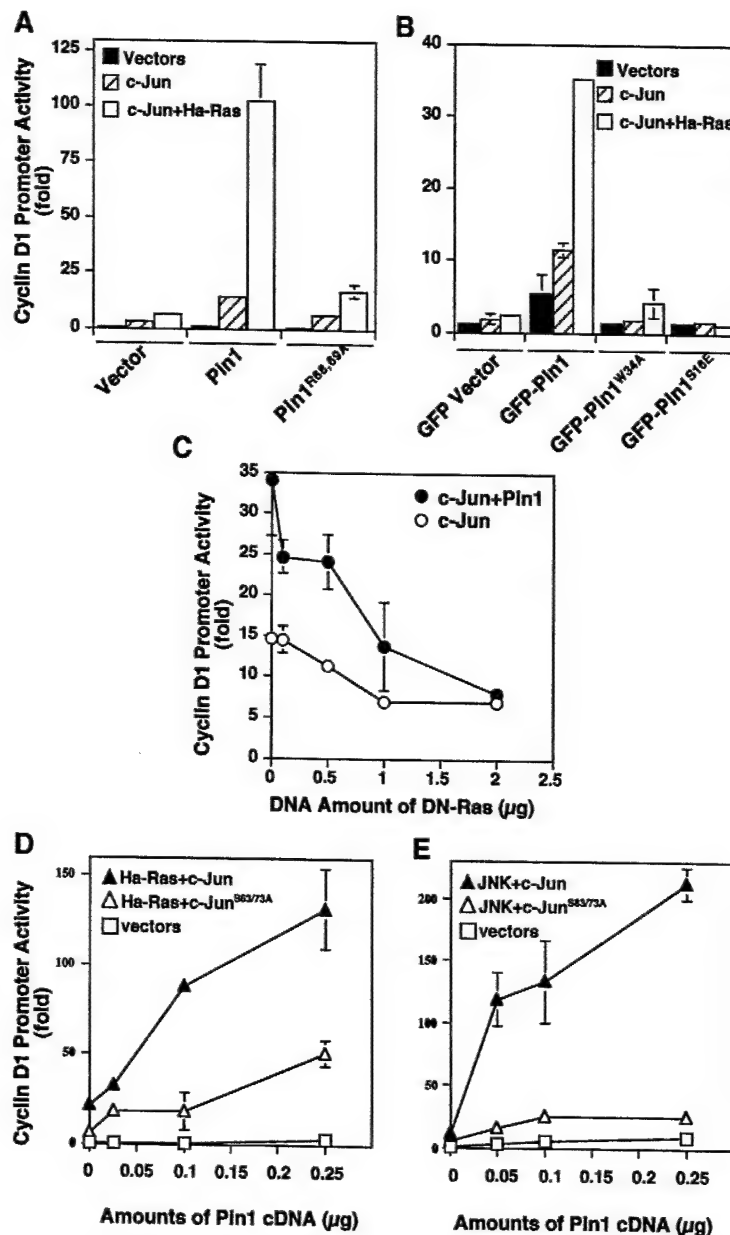
Fig. 6. The effects on the transcriptional activity of c-Jun depend on the phosphoprotein-binding and PPIase activity of Pin1 as well as phosphorylation of c-Jun on Ser$^{63/73}$. (**A**) Abolishing the Pin1 effect by inactivating its PPIase activity. HeLa cells were co-transfected with vectors, c-Jun, or c-Jun + Ha-Ras, and Pin1 or its PPIase-negative mutant Pin1^{R68,69A}, and then subjected to luciferase assay. Pin1^{R68,69A} fails to isomerize phosphorylated Ser/Thr-Pro bonds (Yaffe *et al.*, 1997). (**B**) Abolishing the Pin1 effect by inactivating its phosphoprotein-binding activity. HeLa cells were co-transfected with vectors, c-Jun, or c-Jun + Ha-Ras and green fluorescent protein (GFP)–Pin1 or its WW domain point mutants, and then subjected to luciferase assay. GFP–Pin1^{W34A} and GFP–Pin1^{S16E} did not bind phosphoproteins, as shown (Lu *et al.*, 1999b). Note that GFP fusion proteins were used because the WW domain Pin1 mutants were not stable in cells, but they were stable as GFP fusion proteins, although expressed at reduced levels (data not shown). Although the absolute maximal luciferase activity was not as high as in other experiments, which is likely to be due to lower levels of GFP fusion proteins being expressed, the overall trends were the same. (**C**) Inhibiting the ability of Pin1 to increase the c-Jun activity by DN-Ras. Cells were co-transfected with c-Jun or c-Jun + Pin1, and increasing amounts of DN-Ras, and then subjected to the luciferase assay. (**D** and **E**) Abolishing the cooperative effect between Pin1 and Ha-Ras or activated JNK by mutating c-Jun phosphorylation sites Ser$^{63/73}$. HeLa cells were co-transfected with various amounts of Pin1, c-Jun or c-Jun$^{S63/73A}$ construct, and Ha-Ras (D) or activated JNK (E) and then subjected to the luciferase assay.

the same target c-Jun. However, when the AP-1 site mutant cyclin D1 promoter was used in the same assay, only ≤10% of the transactivation measured for the wild-

type promoter was observed (Figure 5E and F), indicating that transactivation of the cyclin D1 promoter by c-Jun, activated by Pin1, JNK or Ras, is dependent on the intact

AP-1-binding site. These results indicate that Pin1 cooperates either with activated JNK or oncogenic Ras to dramatically activate the cyclin D1 promoter. These cooperative effects are expected because Pin1 can regulate the transcriptional activity of c-Jun only after it has been phosphorylated by them.

To examine whether endogenous Pin1 is important for Ha-Ras to increase the transcriptional activity of c-Jun, we used Pin1^{AS} to reduce cellular Pin1 levels (Figure 3B). When c-Jun and Ha-Ras were co-transfected with different concentrations of the Pin1^{AS} construct, the transcriptional activity of c-Jun decreased significantly in a concentration-dependent manner (Figure 5A), indicating that inhibiting endogenous Pin1 decreases the ability of phosphorylated c-Jun to activate the cyclin D1 promoter. These results indicate that Pin1 cooperates with Ha-Ras or activated JNK to increase the activity of c-Jun toward the cyclin D1 promoter.

Pin1 contains a WW domain and a PPIase domain, which bind and isomerize specific pSer/Thr-Pro motifs, respectively, and both these activities are normally required for Pin1 to modulate the function of its phosphoprotein substrates, such as Cdc25C and tau (Ranganathan *et al.*, 1997; Yaffe *et al.*, 1997; Shen *et al.*, 1998; Lu *et al.*, 1999a,b). To examine whether only one, or both, of these activities is required for Pin1 to modulate the activity of c-Jun we used Pin1 mutants, Pin1^{R68,69A}, Pin1^{W34A} and Pin1^{S16E}, which contain mutations at the key residues either in the PPIase domain (R68, R69) or the WW domain (W34 or S16), and fail to isomerize pSer/Thr-Pro bonds or to bind phosphoproteins (including c-Jun; data not shown), respectively (Shen *et al.*, 1998; Lu *et al.*, 1999b; Zhou *et al.*, 2000). In contrast to wild-type protein, these Pin1 mutants neither increased the transcriptional activity of c-Jun towards the cyclin D1 promoter nor cooperated with Ha-Ras to activate c-Jun (Figure 6A and B). Neither did the mutants affect the levels of c-Jun phosphorylation (data not shown). These results indicate that both phosphoprotein-binding and phosphorylation-specific isomerase activities of Pin1 are required for its ability to modulate the activity of c-Jun.

The above results suggest that Pin1 may increase the activity of c-Jun by binding and isomerizing its pSer/Thr-Pro motifs, as it does to Cdc25C and tau (Shen *et al.*, 1998; Lu *et al.*, 1999a; Zhou *et al.*, 2000). In this case, down-regulation of the Ras-dependent phosphorylation of c-Jun should reduce the effect of Pin1 on c-Jun, and mutations of the c-Jun phosphorylation sites that Pin1 binds to should abolish the Pin1 effect. To examine the first assumption, we co-transfected cells with Pin1, c-Jun and DN-Ras to examine the effect of DN-Ras on the ability of Pin1 to activate the cyclin D1 promoter. DN-Ras reduced both phosphorylation of c-Jun on Ser$^{63/73}$ and the ability of Pin1 to bind c-Jun (Figure 4A–D). Indeed, DN-Ras not only inhibited the ability of c-Jun to activate the cyclin D1 promoter, as shown previously (Albanese *et al.*, 1995), but also inhibited the ability of Pin1 to enhance the activity of c-Jun 5- to 7-fold (Figure 6C). These results suggest that the Ras-dependent phosphorylation of c-Jun is important for the Pin1 function on c-Jun. To examine the second assumption, we used the mutant c-Jun$^{S63/73A}$, which failed to bind Pin1 (Figure 4E and F). Pin1 almost completely failed to cooperate either with

activated JNK or oncogenic Ha-Ras to increase the ability of c-Jun$^{S63/73A}$ to induce the cyclin D1 promoter (Figure 6D and E), indicating that phosphorylation of c-Jun on Ser$^{63/73}$ is essential for Pin1 to induce the cyclin D1 promoter. These results indicate that phosphorylation of c-Jun on Ser$^{63/73}$, induced by the Ras-dependent signaling pathway, is essential for Pin1 to increase transcription of the cyclin D1 promoter. Thus, Pin1 binds phosphorylated c-Jun and potentiates its transcriptional activity towards cyclin D1 in response to activation of Ras or JNK.

Discussion

Previous studies have demonstrated that depletion of Pin1 induces apoptosis and is also observed in neuronal cell death in Alzheimer's disease (Lu *et al.*, 1996, 1999a). We show here the striking overexpression of Pin1 in a large fraction of breast cancers. Furthermore, Pin1 levels correlate significantly with the grade of the breast tumors according to Bloom and Richardson's classification system, although the relationship between Pin1 levels and the prognosis of cancer patients remains to be determined. Consistent with our findings is the observation that Pin1 is one of the genes that are most drastically suppressed by up-regulation of Brca1, as detected in cDNA array screening and northern analysis (MacLachlan *et al.*, 2000). In addition, the level of Pin1 in breast cancer cell lines is much higher than that in either normal or non-transformed mammary epithelial cells. Although further studies are needed to elucidate the mechanisms leading to overexpression of Pin1, these results demonstrate for the first time that Pin1 is up-regulated markedly in many human tumor samples.

The significance of Pin1 overexpression in cancer is further substantiated by our findings that Pin1 cooperates with activated JNK or Ha-Ras in increasing the transcriptional activity of phosphorylated c-Jun to activate the cyclin D1 promoter. Overexpression of cyclin D1 is found in 50% of patients with breast cancer (Bartkova *et al.*, 1994; Gillett *et al.*, 1994). Furthermore, overexpression of cyclin D1 contributes to cell transformation (Hinds *et al.*, 1994), whereas inhibition of cyclin D1 expression by antisense expression causes growth arrest of tumor cells (Schrump *et al.*, 1996; Arber *et al.*, 1997; Driscoll *et al.*, 1997; Kornmann *et al.*, 1998). Disruption of the cyclin D1 gene in mice blocks the proliferation of breast epithelial cells and reduces tumor development in response to Ha-Ras (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998). These results indicate that cyclin D1 plays an important role during oncogenesis, especially during Ras-mediated tumorigenesis (Rodriguez-Puebla *et al.*, 1999). Oncogenic Ras induces the cyclin D1 promoter via its AP-1 site (Albanese *et al.*, 1995). Although the AP-1 complex is composed of the c-Jun and c-Fos proteins, c-Jun is the most potent transactivator in the complex (Angel *et al.*, 1989; Chiu *et al.*, 1989; Abate *et al.*, 1991) and is elevated in Ha-Ras-transformed cells, in which c-Fos is down-regulated (Thomson *et al.*, 1990; Binetruy *et al.*, 1991). In addition to the regulation of protein levels, the activity of c-Jun is enhanced by phosphorylation induced by growth factors, oncogenic proteins, or stress conditions. Although different pathways may be involved, they eventually lead to activation of JNKs, which
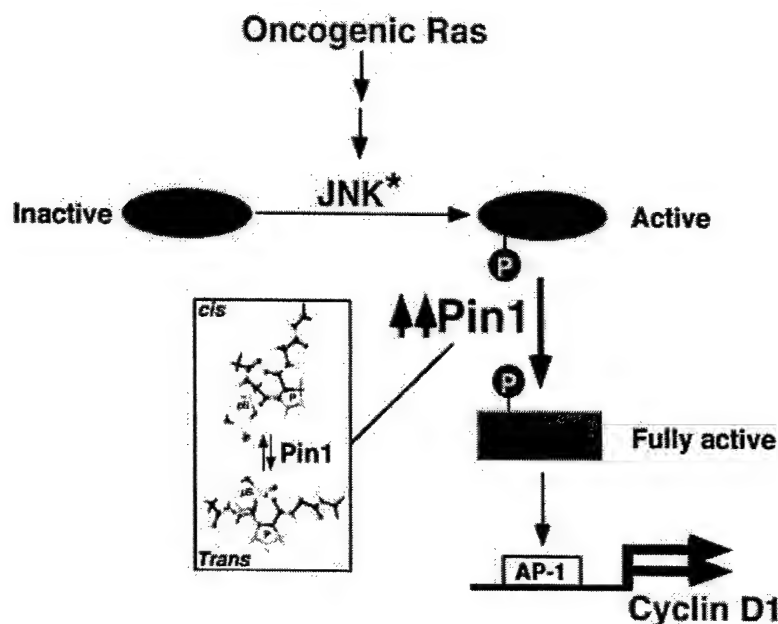
Fig. 7. Role of Pin1 in regulating the transcriptional activity of phosphorylated c-Jun towards the cyclin D1 promoter. Oncogenic Ha-Ras activates JNKs, which phosphorylate c-Jun on two critical amino terminal Ser-Pro motifs, enhancing its transcriptional activity. Pin1 is up-regulated in breast cancer and functions as a potent regulator of phosphorylated c-Jun to induce cyclin D1 expression, presumably by altering the conformation of the phosphorylated Ser-Pro motifs (insert). Double arrows, up-regulation; the asterisk indicated the activated form of proteins.

phosphorylate c-Jun on two critical N-terminal Ser-Pro motifs ($S^{63/73}$-P) and enhance its transcriptional activity (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Hunter and Karin, 1992; Derijard *et al.*, 1994; Hinds *et al.*, 1994; Albanese *et al.*, 1995, 1999; Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Whitmarsh and Davis, 1996; Karin *et al.*, 1997; Robles *et al.*, 1998; Bakiri *et al.*, 2000). Thus, phosphorylation of c-Jun on $Ser^{63/73}$-Pro is a key regulatory mechanism that converts inputs from various signaling pathways into changes in gene expression. However, it has not been described previously whether the activity of phosphorylated c-Jun is further regulated after phosphorylation.

We have found that Pin1 not only binds phosphorylated c-Jun, but also dramatically increases its ability to activate the cyclin D1 promoter in cooperation either with activated JNK or oncogenic Ha-Ras. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun, indicating that endogenous Pin1 is also required for the optimal activation of c-Jun. The significance of this Pin1-dependent regulation is further substantiated by our findings that up-regulation of Pin1 not only correlates with cyclin D1 overexpression in breast cancer tissues, but also induces cyclin D1 expression in breast cancer cell lines. Thus, Pin1 is a potent modulator of phosphorylated c-Jun in inducing cyclin D1 expression, presumably by regulating the conformation of the phosphorylated Ser-Pro motifs in c-Jun (Figure 7). The importance of Pin1 in the regulation of cyclin D1 expression has been further supported by our recent identification of cyclin D1 as one of the Pin1-induced genes in breast cancer cells in the differential display screen (Ryo *et al.*, 2001), and by our phenotypic analysis of Pin1-deficient

mice (Y.-C.Liou, A.Ryo, H.K.Huang, P.J.Lu, F.Fujimori, T.Uchida, R.Bronson, T.Hunter and K.P.Lu, submitted). Although Pin1$^{-/-}$ mice have previously been shown to develop normally (Fujimori *et al.*, 1999), we have uncovered that they display a range of cell proliferative abnormalities, including decreased body size, retinal degeneration and neurological abnormalities. Moreover, in Pin1-deficient adult females, the breast epithelial compartment failed to undergo the massive proliferative changes caused by pregnancy (Y.-C.Liou, A.Ryo, H.K.Huang, P.J.Lu, F.Fujimori, T.Uchida, R.Bronson, T.Hunter and K.P.Lu, submitted). Significantly, many features of these Pin1-deficient mice, such as retinal degeneration and mammary gland impairment, are also characteristic of cyclin D1-deficient mice (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Moreover, cyclin D1 levels were significantly reduced in Pin1-deficient retina and breast epithelial cells from pregnant mice (Liou *et al.*, submitted). These results provide the genetic evidence for an essential role of Pin1 in maintaining cell proliferation and cyclin D1 expression, and further support a role of Pin1 in oncogenesis. Abnormal activation of the Ras-dependent signaling pathway and cyclin D1 overexpression are a common and critical mechanism during the development of many malignancies, such as breast, skin and colon cancer (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Rodriguez-Puebla *et al.*, 1999). Indeed, Pin1 is significantly overexpressed in many of these human tumors (G.M.Wulf and K.P.Lu, unpublished data), suggesting that it plays a positive role for cell proliferation during oncogenesis (Figure 7).

In summary, our results show that Pin1 is strikingly overexpressed in human breast cancer tissues, and

cooperates with activated Ras signaling in increasing c-Jun transcriptional activity towards the cyclin D1 gene. Given the well established role of activated Ras signaling and cyclin D1 overexpression during onco-genesis, our study suggests that overexpression of Pin1 may promote tumor growth. In addition, since inhib-ition of the Pin1 enzymatic activity triggers tumor cells to enter apoptosis, overexpressed Pin1 may act as a novel anti-cancer target.

Materials and methods

Analysis of protein and mRNA levels in patient samples

Fifty-one cancerous and 10 normal breast tissue specimens were randomly selected. The malignancy of infiltrating carcinomas was scored according to Bloom and Richardson's classification system (Bloom and Richardson, 1957). Tissue from the core of the tumor was snap frozen in liquid nitrogen and pulverized using a Microdismembrator (Braun). About 10 μg of the pulverized tissues were resuspended in 100 μl of SDS sample buffer. Immunoblotting with anti-Pin1, anti-cyclin D1, anti-Her2/neu and anti-actin antibodies was performed as described (Shen et al., 1998; Lu et al., 1999a), as was immunohistochemistry using anti-Pin1 polyclonal or monoclonal antibodies (Lu et al., 1999a). Levels of Pin1 and actin were semi-quantified using Imagequant, as described (Lu et al., 1999a). mRNA was isolated using the Trizol reagent (Gibco) and cDNA was synthesized using Superscript (Gibco). Twenty-five nanograms of cDNA were used per real-time PCR run with primers specific for cyclin D1, and GAPDH as an internal control. All real-time PCR runs were performed in duplicate and analyzed according to the manufactur-er's instructions (Applied Biosystems). The significance of the differ-ences in Pin1 levels between clinical and pathological categories was analyzed using the Kruskal–Wallis test (Glantz, 1997). The Pearson correlation coefficients were obtained using the SAS software (Release 6.12; SAS Institute Inc., Cary, NC).

Determination of Pin1 levels and the effects of Pin1 on cyclin D1 expression in cell lines

The levels of Pin1 in normal (76N), spontaneously immortalized but not transformed (184B5 and MCF10), and transformed (MCF7, T47D, MDAMB435 and HCC1937) mammary epithelial cell lines were determined by subjecting total cellular proteins to immunoblotting analysis with anti-Pin1 polyclonal antibodies. To examine the nature of the double band, a tumor lysate was incubated at 30°C for 60 min in the presence of 100 nM okadaic acid (Sigma), PP1 and PP2A (Upstate Biotechnology) or CIP. To examine the effects of Pin1 on cyclin D1 expression, Pin1 cDNA was subcloned into pcDNA3 vector (Invitrogen) and transfected into MCF7, T47D or HeLa cells for 36 h, followed by determining the level of Pin1 and cyclin D1 by immunoblotting analysis with anti-Pin1 and anti-cyclin D1 antibodies, respectively, as described (Lu et al., 1996; Shen et al., 1998), and cyclin D1 mRNA by real-time PCR, as described above.

Determination of the Pin1-c–Jun interaction

To examine the interaction between Pin1 and phosphorylated c-Jun, HeLa cells were co-transfected with c-Jun or c-Jun$^{S63/73A}$ and the oncogenic Ha-Ras, constitutively active JNK, DN-Ras or the control vector for 24 h. The cells were lysed in a lysis buffer containing 1% Triton X-100, and the supernatants incubated with 10 μl of agarose beads containing various GST–Pin1 proteins or control GST for 2 h at 4°C. The precipitated proteins were washed five times in the buffer containing 1% Triton X-100 before being subjected to immunoblotting analysis using antibodies against c-Jun or c-Jun phosphorylated on Ser$^{63/73}$ (New England Biolabs), as described (Yaffe et al., 1997; Shen et al., 1998; Lu et al., 1999a,b). For co-immunoprecipitation, we used anti-c-Jun polyclonal antibodies (Santa Cruz) and unrelated polyclonal antibodies (Pericentrin antibodies) as a control. The pre-cleared lysates were incubated for 2 h with the respective antibodies, and the immune complexes were collected with protein A beads (Sigma) and subjected to immunoblotting with anti-Pin1 or anti-c-Jun antibodies. The ability of the Pin1 WW domain and PPIase domain mutants to bind phosphoproteins (MPM-2 or c-Jun) and to isomerize pSer/Thr-Pro bonds were determined, as described (Yaffe et al., 1997; Lu et al., 1999a,b).

Promoter reporter assays

Various cyclin D1–luciferase reporter constructs, c-Jun and Ras constructs were gifts from R.Pestell (Albert Einstein College of Medicine), M.Karin (University of California at San Diego) and L.Feig (Tufts University), respectively, and have been confirmed by DNA sequencing. Luciferase reporter constructs for TK, c-fms and MMTV were purchased. Superfect (Qiagen) was used for transfections. Reporter gene assays were performed with the Dual-luciferase reporter assay system (Promega) at 24–36 h after transfection. One nanogram of pRL-TK (Promega) Renilla luciferase was co-transfected in each sample as an internal control for transfection efficiency. Expression of all transfected genes was confirmed by immunoblotting analysis with the respective antibodies. The amounts of DNA used in transfection were carefully titrated for each construct; typically, only ~50 ng of each DNA were used, with exceptions indicated in the text. The activity of the reporter luciferase was expressed relative to the activity in control vector-transfected cells, which was defined as 1. Similar results were obtained in at least three different experiments. All results are expressed as $\bar{x} \pm SD$ of independent duplicate cultures. Since Pin1^{AS} induces mitotic arrest and apoptosis at 48–72 h after transfection (Lu et al., 1996), all experiments with Pin1^{AS} were performed before 36 h, when no significant apoptotic cells were observed, as described previously (Lu et al., 1996).

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Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC

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Phosphorylation on a serine or threonine residue preceding proline (Ser/Thr-Pro) is a key regulatory mechanism, and the conformation of certain phosphorylated Ser/Thr-Pro bonds is regulated specifically by the prolyl isomerase Pin1. Whereas the inhibition of Pin1 induces apoptosis, Pin1 is strikingly overexpressed in a subset of human tumours. Here we show that Pin1 regulates β-catenin turnover and subcellular localization by interfering with its interaction with adenomatous polyposis coli protein (APC). A differential-display screen reveals that Pin1 increases the transcription of several β-catenin target genes, including those encoding cyclin D1 and c-Myc. Manipulation of Pin1 levels affects the stability of β-catenin *in vitro*. Furthermore, β-catenin levels are decreased in Pin1-deficient mice but are increased and correlated with Pin1 overexpression in human breast cancer. Pin1 directly binds a phosphorylated Ser-Pro motif next to the APC-binding site in β-catenin, inhibits its interaction with APC and increases its translocation into the nucleus. Thus, Pin1 is a novel regulator of β-catenin signalling and its overexpression might contribute to the upregulation of β-catenin in tumours such as breast cancer, in which APC or β-catenin mutations are not common.

U pregulation of the oncogenic transcriptional activator β-catenin has a pivotal role in the development of cancer[1-3]. One of the key β-catenin regulators is adenomatous polyposis coli protein (APC), which is encoded by the tumour-suppressor gene that is mutated in familial adenomatous polyposis coli[4-9]. The tumour-suppressing activity of APC largely involves controlling the nuclear accumulation of the oncogenic transcriptional activator β-catenin[4-10]. The stable overexpression of β-catenin caused by mutations in APC or β-catenin leads to an accumulation of β-catenin in the nucleus, where it induces a set of genes critical for the development of cell transformation and cancer, including those encoding cyclin D1, c-Myc, fibronectin and peroxisome-proliferator-activated receptor-δ (PPAR-δ)[11-16]. There are two major mechanisms by which APC modulates the concentration of β-catenin in the nucleus. First, APC binds and assembles β-catenin into a multiple protein complex, including glycogen synthase kinase-3β (GSK-3β) and trigger the degradation of β-catenin[17,18]. However, the activation of Wnt signalling inhibits the phosphorylation of β-catenin by GSK-3β, resulting in the stabilization of β-catenin in the cytoplasm and nucleus[1-3,17,19]. Second, APC binds the nuclear β-catenin and exports it to the cytoplasm for degradation[20-22]. Thus, a crucial step in the APC-mediated regulation of β-catenin is the interaction between them. Indeed, this interaction is often disrupted in many mutations of APC in cancer[5,6]. Therefore, disruption of the interaction between APC and β-catenin has a pivotal role in oncogenesis. However, it is not known whether this interaction can be regulated.

Although genetic mutations of APC or β-catenin are often found in some tumours, such as colon cancer[1-3], they are rarely observed in others such as breast cancer[23-25]. However, compelling evidence has indicated a crucial role for signalling by β-catenin in the tumorigenesis of breast cancer[23-26]. Furthermore, β-catenin levels are significantly upregulated and are correlated with poor prognosis, acting as a strong and independent prognostic factor in
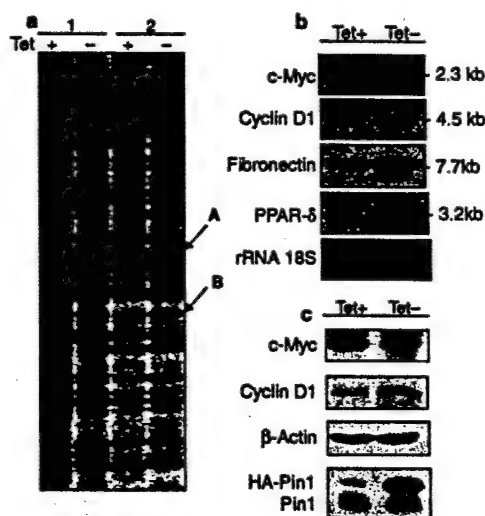
Figure 1 **Activation of genes downstream of those encoding β-catenin/TCF by overexpression of Pin1. a,** Representative results of the differential display screen. Pin1 was induced in the breast-cancer cell line MCF-7 with the Tet-Off gene expression system (Clontech). MCF-7 cells were cultured in the presence (Tet+) or absence (Tet–) of tetracycline for 24 h and the isolated RNA was subjected to a differential display screen. The results obtained from the two respective combinations of extended primers are shown; the bands were excised for further analysis are marked with arrowheads: A, upregulated CD97; B, upregulated PPAR-δ; C, a down-regulated unknown gene. **b, c,** Total RNA and cell lysates were prepared from the same cells as described in **a** and subjected to northern blotting (**b**) or immunoblotting analysis (**c**).
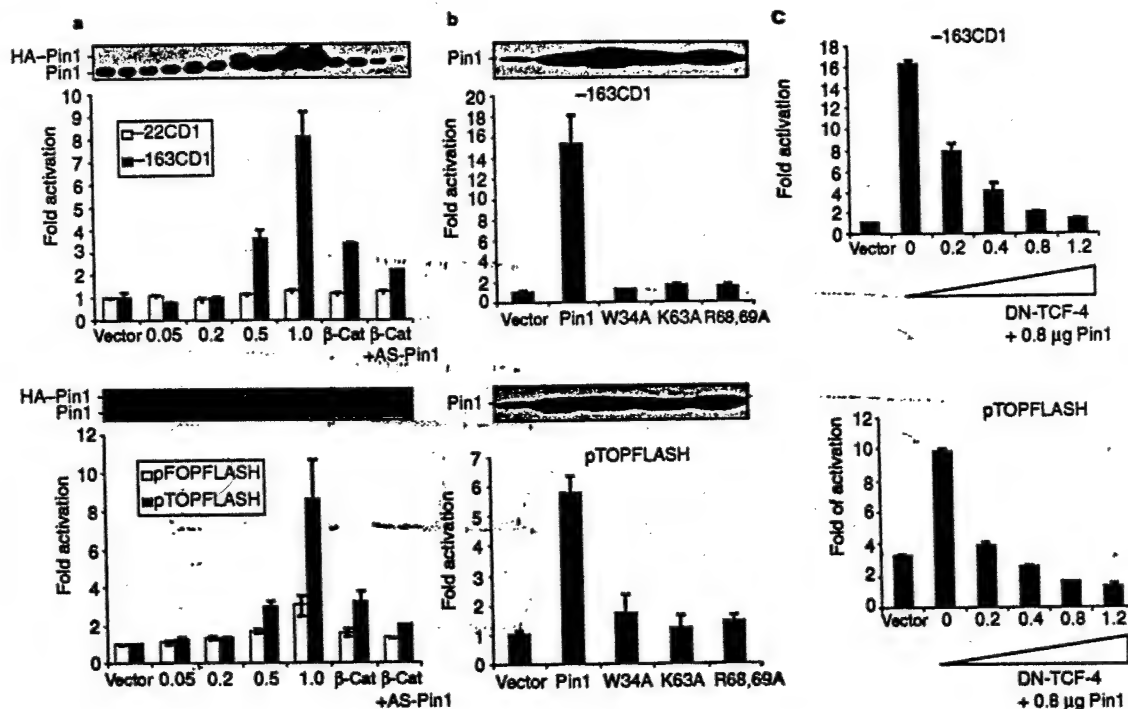
Figure 2 **Pin1 transactivates genes downstream of those encoding β-catenin/TCF through TCF sites.** a, HeLa cells were transfected with different concentrations of Pin1 and various reporter constructs as indicated, followed by an assay of the luciferase activity. pRL-TK *Renilla* luciferase reporter construct was co-transfected in each sample to normalize for transfection efficiency. The activity of the reporter luciferase was expressed relative to the activity in control-vector-transfected cells, which is defined as 1.0. −22CD1, the cyclin D1 minimal promoter region from −22 to +14; −163CD1, the cyclin D1 minimal promoter region from −163 to +14 containing three TCF-binding sites; pTOPFLASH, three copies of the optimal TCF motif CCTTTGATC; pFOPFLASH, three copies of the mutant motif CCTTTGGCC. Expression of endogenous Pin1 and exogenous haemagglutinin (HA)-labelled Pin1 was detected by western blot analysis with a monoclonal antibody against Pin1; this is shown at the top of the panels. All experiments were repeated in triplicate and are expressed as means ± s.d. b, HeLa cells were co-transfected with Pin1, its WW-domain mutant (W34A) or its PPIase-domain mutants (K63A and R68,69A), and −163CD1 or pTOPFLASH luciferase reporter construct, followed by the luciferase assay. c, HeLa cells were co-transfected with Pin1, −163CD1 or pTOPFLASH luciferase reporter construct and increasing concentrations of dominant-negative TCF-4 (DN-TCF-4), followed by the luciferase assay.

Upregulated genes	Downregulated genes
5-aminolevulinate synthase	Serine/threonine protein kinase gsk
CD97	α-2-actinin
BIP/GRP78	Tu translation-elongation factor (p43)
PPAR-delta*	Platelet-activating factor acetylhydrolase Ib-γ
SRY (sex-determination Y)-box 22 (SOX22)	Eukaryotic translation-initiation factor 4E binding protein 1
IFN-inducible γ2-protein	
Stress-inducible phosphoprotein 1 (STIMP1)	
Phosphoenolpyruvate carboxykinase 1 (PCK1)	
Fibronectin*	
C-Myc*	
Profilin	
Cyclin D1*	

*β-catenin/Tcf-target genes

human breast-cancer patients[24]. Additional mechanisms can therefore be used to upregulate β-catenin levels in breast cancer. We

have recently shown that Pin1 is strikingly overexpressed in human breast cancer and some other tumours[27]. Furthermore, Pin1 levels are correlated with the tumour grade and with cyclin D1 levels in breast-cancer tissues[27]. These results indicate that Pin1 might have a role in oncogenesis.

Pin1 is a peptidyl-prolyl *cis–trans* isomerase (PPIase) that isomerizes only phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) peptide bonds[28–31]. Pin1-catalysed isomerization regulates the conformation of a subset of phosphoproteins such as Cdc25C and the microtubule-associated protein tau, thereby affecting their activity and/or protein–protein interactions[30–35]. Interestingly, the depletion of Pin1 in tumour cells induces apoptosis[28,36] and also contributes to neuronal death in Alzheimer's disease[34]. Conversely, Pin1 is overexpressed in many human tumours such as breast and prostate cancer, and increases the transcriptional activity of c-Jun towards the cyclin D1 promoter[27]. These results indicate that Pin1 overexpression might promote tumour cell growth by altering gene expression.

To test this hypothesis, we here used a differential display screen and found that Pin1 transactivated several β-catenin target genes. Furthermore, Pin1 increased the stability of β-catenin in cells, which is substantiated by the findings that β-catenin was increased and correlated with Pin1 in breast cancer tissues, but drastically decreased in Pin1-deficient mouse tissues. Moreover, Pin1 stabilized and subsequently increased the nuclear fraction of β-catenin by preventing its interaction with APC. These findings uncover a novel mechanism for regulating signalling by β-catenin and support a role for Pin1 in tumorigenesis.
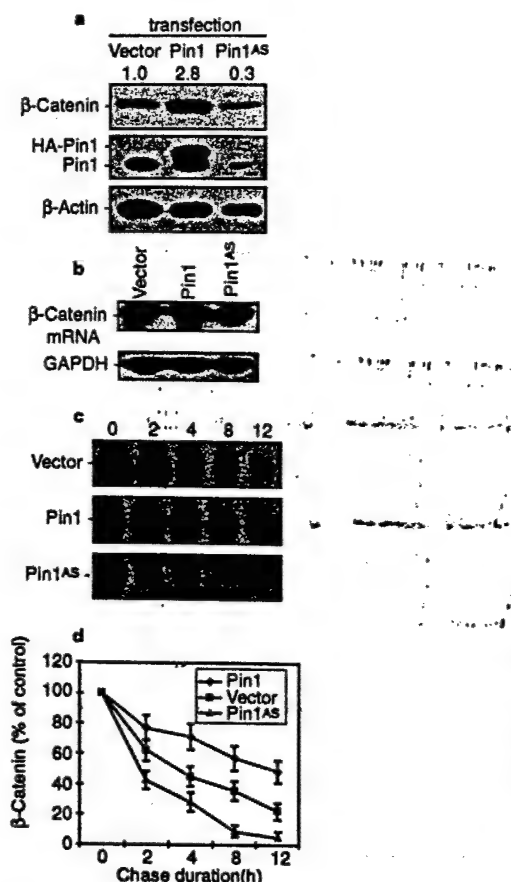
Figure 3 **Pin1 stabilizes cellular β-catenin. a,** HeLa cells were transfected with 0.5 μg of a sense (Pin1) or antisense Pin1 (Pin1^AS) expression construct or control vector and harvested 48 h after transfection, followed by immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-β-actin antibodies. Numbers above the gel image indicate the fold induction of endogenous β-catenin normalized with β-actin. **b,** Total RNA was extracted from the same cells as those in a and subjected to northern blot analysis. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. **c, d,** Subconfluent cells were transfected with a sense (Pin1) or antisense Pin1 (Pin1^AS) expression construct or control vector. After 24 h, cells were pulse-labelled with [35S]methionine for 1 h and chased for the durations indicated (c). Labelled β-catenin was detected by immunoprecipitation with anti-β-catenin, followed by SDS–PAGE and autoradiography. The radioactivity of immuno-precipitated β-catenin was quantified with a PhosphorImager and normalized to the 0 h point (d). Results shown are means ± s.d. for three independent experiments.

Figure 4 **β-Catenin levels are decreased in Pin1-deficient mice but increased in breast-cancer samples overexpressing Pin1. a,** Different issues of wild-type (+/+) or Pin1 knockout mice (–/–) were homogenized and then subjected to immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-α-tubulin antibodies. **b,** Normal breast and breast-cancer tissues were homogenized and subjected to immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-α-tubulin antibodies. Normal 1–4, non-neoplastic breast; cancer 1 and 2, Bloom and Richardson grade III; cancer 3–5: grade II. **c,** The correlation between Pin1 and β-catenin protein levels in malignant and normal breast tissues. Immunoblot analysis was performed on 45 breast-cancer and 6 normal tissue samples, as in b. The densities of bands were quantified by NIH Image, normalized with β-actin and plotted. The correlation was tested by a linear regression analysis ($r^2 = 0.69$, $P < 0.01$).

Results

Pin1 enhances the expression of genes downstream of those encoding β-catenin/T-cell factor (TCF). To examine the role of Pin1 overexpression in human breast cancer, we used a newly developed differential display method[37,38] to identify genes and signalling pathways regulated by Pin1. By comparing gene expression patterns of about 10,000 complementary DNA fragments between Pin1-overexpressing breast-cancer MCF-7 cells and control MCF-7 cells (see Fig. 1a), we selected and sequenced 48 cDNA fragments that were obviously expressed differentially between Pin1-induced and non-induced conditions. From these 48 clones we reproducibly identified 17 known genes whose expression was upregulated or downregulated by the overexpression of Pin1 (Table 1). Interestingly, a database search revealed that four of the twelve upregulated genes identified were downstream of those encoding β-catenin/TCF, which included those for cyclin D1, c-Myc, PPAR-δ

and fibronectin[15–16]. To confirm that these genes are indeed induced by Pin1, we isolated messenger RNAs and subjected them to northern blotting analysis. Figure 1b shows that mRNA levels of these genes were indeed higher in cells expressing Pin1 than those in control cells. Furthermore, immunoblotting analysis with cell lysates from the same cells also showed an enhanced expression of c-Myc and cyclin D1 proteins (Fig. 1c). These results indicate that Pin1 can affect the gene expression pattern by activating the β-catenin/TCF signalling pathway.

To confirm that Pin1 activates the β-catenin/TCF signalling pathway, we used two sets of reporter constructs in a β-catenin/TCF reporter gene assay, as described previously[7,15]. Although Pin1 cDNA had no significant effect on the −163CD1 promoter at low concentrations, as shown previously[27], Pin1 increased the activity of both the −163CD1 and pTOPFLASH promoters in a dose-dependent manner (Fig. 2a). In contrast, Pin1 had no significant effect on the control promoter, that is, on either the −22CD1 promoter or the pFOPFLASH promoter (Fig. 2a). Furthermore, the depletion of endogenous Pin1 by the expression of an antisense Pin1 construct inhibited the ability of β-catenin to activate the −163CD1 or pTOPFLASH promoter (Fig. 2a). These results demonstrate that Pin1 increases the activity of both the cyclin D1 and pTOPFLASH promoters.

Both the binding and isomerizing activities of Pin1 are normally required for Pin1 to regulate the function of its substrates[29,30,32–34]. To examine whether any one or both of these activities are required for Pin1 to modulate the activity of the −163CD1 or pTOPFLASH promoter, we used Pin1 mutants Pin1^W34A, which fails to bind to phosphoprotein, and Pin1^K63A and Pin1^R68,69A, which fail to bind phosphoproteins or isomerize pSer/Thr-Pro bonds[31–33]. As shown in Fig. 2b, in contrast to wild-type Pin1, neither the WW-domain mutant (Pin1^W34A) nor the PPIase mutants (Pin1^K63A and Pin1^R68,69A) increased the activity of the −163CD1 or pTOPFLASH promoter.
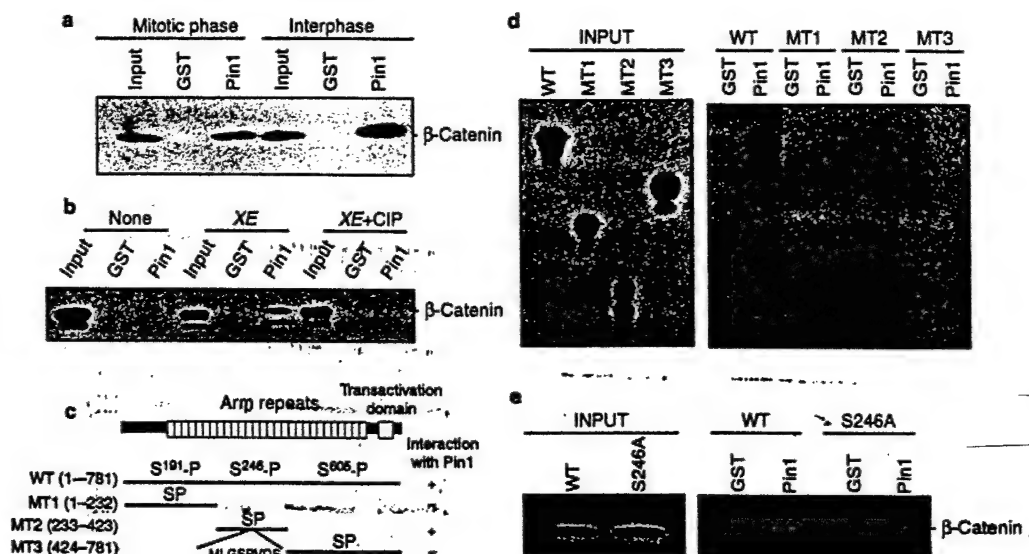
Figure 5 Pin1 binds β-catenin phosphorylated on the Ser 246-Pro motif in the middle of the Armadillo repeat domain. a, Glutathione–agarose beads containing GST or GST–Pin1 were incubated with extracts of HeLa cells in interphase or undergoing mitosis. After washing, binding proteins were subjected to immunoblotting analysis with anti-β-catenin antibodies. b, β-Catenin protein was synthesized by transcription and translation in vitro in the presence of [35S]methionine. The labelled protein was incubated with control buffer (None), Xenopus extracts (XE) or Xenopus extracts followed by treatment with calf intestinal alkaline phosphatase

(XE+CIP). These proteins were separated on SDS-containing gels either directly (input) or after GST pulldown experiments with glutathione beads containing GST or GST–Pin1. c, Schematic representation of wild-type (WT) β–catenin and its truncation mutants. d, Wild-type β-catenin and its truncation mutants were labelled with [35S]methionine, incubated with Xenopus extracts and then subjected to GST pull-down experiments. e, β-Catenin and its site-directed S246A mutant were labelled with [35S]methionine, incubated with Xenopus extracts and then subjected to GST pulldown experiments.
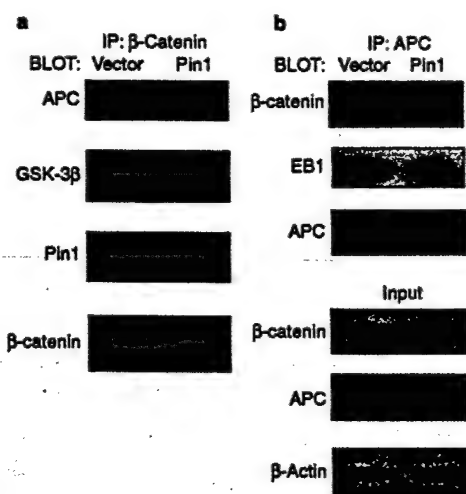
Figure 6 Pin1 selectively inhibits the interaction between β-catenin and APC. HeLa cells were transfected with Pin1 expression construct or control vector for 24 h, and lysates were subjected to immunoprecipitation (IP) with anti-β-catenin (a) or anti-APC antibody (b). Immunoprecipitates were subjected to SDS–PAGE and immunoblotting analysis with various antibodies as indicated. Experiments were repeated several times with similar results.

These results indicate that both phosphoprotein-binding and phosphorylation-specific isomerase activities of Pin1 are required for activation of the β-catenin-dependent transcription.

To examine whether these effects of Pin1 on the activity of the cyclin D1 and pTOPFLASH promoters are mediated by the TCF-

binding site, we used truncated TCF-4 (DN-TCF4), which has been shown to act as a dominant-negative mutant[7,13]. When co-transfected into cells with Pin1, the dominant-negative TCF-4 mutant decreased the ability of Pin1 to increase the −163CD1 or pTOPFLASH promoter activity in a dose-dependent manner (Fig. 2c). These results indicate that Pin1 enhances the ability of the β-catenin/TCF signalling pathway to activate its downstream target genes through the TCF sites, which is consistent with the findings that Pin1 did not activate the pFOPFLASH and −22CD1 promoters.

Pin1 stabilizes β-catenin by post-translational regulation. The fact that Pin1 increases the transcription of the genes downstream of those encoding β-catenin/TCF indicates that Pin1 might affect the protein levels of β-catenin. To examine this possibility, we examined the effect of manipulating cellular Pin1 levels on levels of β-catenin protein and mRNA. Figure 3a shows that the overexpression and depletion of Pin1 significantly increased and decreased β-catenin protein levels, respectively. However, no difference in β-catenin mRNA level was observed when Pin1 was overexpressed or depleted (Fig. 3b), indicating that Pin1 does not increase the transcription of the gene encoding β-catenin. To investigate whether these effects of Pin1 on the β-catenin protein levels were due to post-translational regulation, we used a pulse–chase study to analyse the effects of Pin1 on the stabilization of endogenous β-catenin. The metabolic stability of β-catenin was significantly increased in Pin1-overexpressed cells but decreased in Pin1-depleted cells (Fig. 3c). Quantification by PhosphorImager revealed that, in comparison with vector-transfected control cells, the half-life of β-catenin was significantly affected by manipulating cellular concentrations of Pin1, especially at the early time points (Fig. 3d). Taken together, these results indicate that Pin1 increases the levels of β-catenin protein by inhibiting its degradation.

The level of β-catenin is downregulated in Pin1 knockout mice but upregulated and correlated with Pin1 overexpression in human breast cancer tissues. To determine the effects of Pin1 on β-catenin levels in vivo, we first examined β-catenin protein levels in tissues
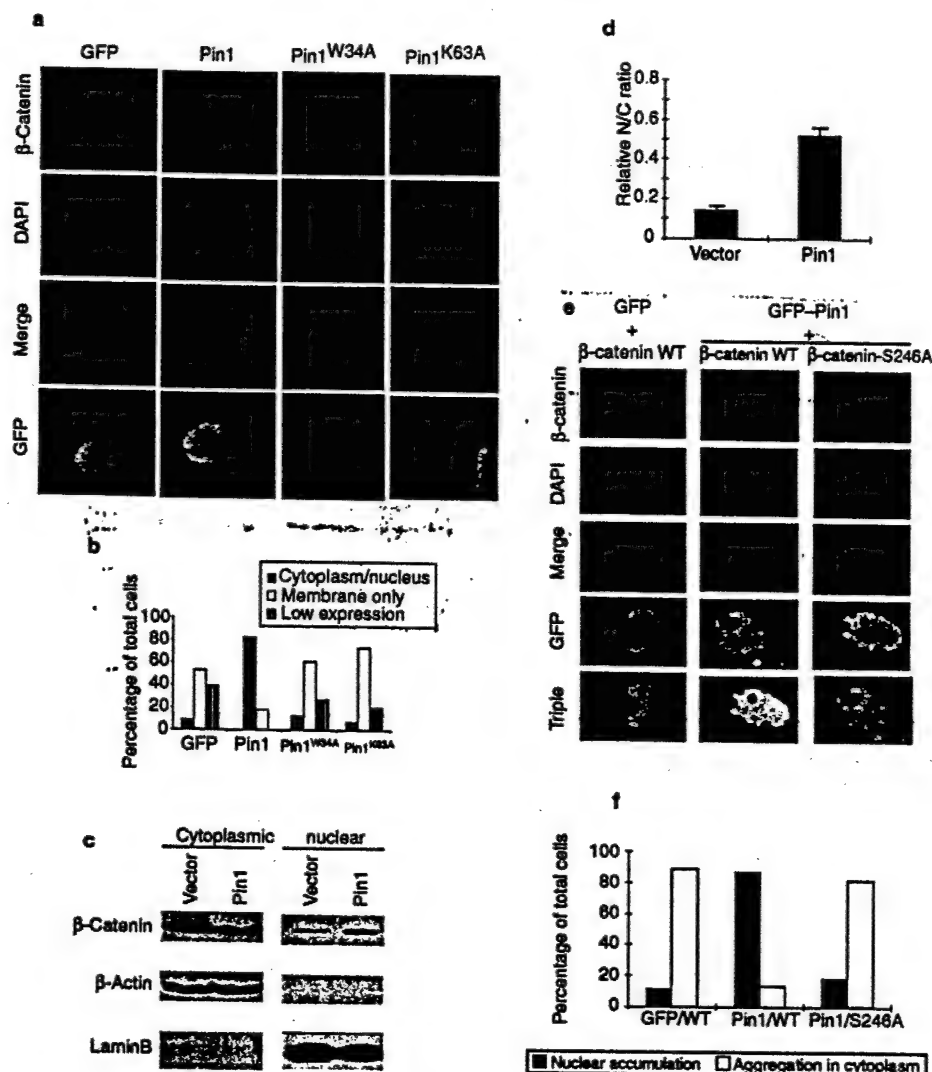
Figure 7 **Pin1 induces nuclear translocation of β-catenin. a, b,** HeLa cells were transfected for 24 h with the construct expressing GFP, GFP–Pin1, its WW domain mutant (Pin1[W34A]) or its PPIase domain mutant (Pin1[K63A]). Cells were fixed and stained with anti-β-catenin antibodies and DAPI to detect endogenous β-catenin and DNA, respectively (a). Localization and expression of β-catenin were scored in 100 transfected cells (b). **c, d,** HeLa cells were transfected for 24 h with Pin1 expression construct or the control vector. Cells were fractionated in hypotonic buffer into the nuclear and cytoplasmic fractions, followed by immunoblotting analy-sis with anti-β-catenin, anti-lamin B or anti-β-actin antibody. Lamin B and β-actin were used as nuclear and cytoplasmic markers, respectively (c). Relative amounts of nuclear and cytoplasmic (N/C) β-catenin were semi-quantified with ImageQuant and normalized with lamin B or β-actin (d). **e, f,** HeLa cells were transfected with GFP–vector and RFP–β-catenin-WT, GFP–Pin1 and RFP–β-catenin or GFP–Pin1 and RFP–β-catenin-S246A for 24 h. Cells were fixed and stained with DAPI and analysed under a fluorescence microscope (e). Localization of β-catenin was scored in 100 transfected cells (f).

of Pin1 knockout mice and in human breast-cancer samples. Pin1 knockout mice have been generated previously and shown to devel-op normally[39]. As expected, Pin1 was not detected in Pin1 knock-out mouse tissues (Fig. 4a). Importantly, amounts of β-catenin protein were decreased significantly in all tissues examined from Pin1 knockout mice, in comparison with those from wild-type mice (Fig. 4a), indicating that the β-catenin level is downregulated in Pin1 knockout mice. This is consistent with our new findings that mice lacking Pin1 demonstrate phenotypes remarkably similar to those in cyclin D1 knockout mice (Y.-C.L., A.R., H. K. Huang, P. J. Lu, R. Bronson, F. Fujimori, T. Uchida, T. Hunter and K.P.L., unpublished results).

We have recently shown that Pin1 is overexpressed in human breast-cancer tissues and its expression level is correlated with tumour grade and cyclin D1 levels[27]. To examine whether Pin1 overexpression is correlated with β-catenin levels in breast-tumour tissues, we determined levels of Pin1 and β-catenin in breast-cancer tissues. Figure 4b shows that both β-catenin and Pin1 were highly overexpressed in breast-cancer tissues in comparison with normal tissues. We performed the same immunoblot analysis and quanti-fied the expression level of both Pin1 and β-catenin in 45 breast-cancer and 6 normal breast tissues. Regression analysis revealed that the levels of β-catenin were significantly correlated with those of Pin1 in these tissues ($r^2 = 0.69$, $P < 0.01$) (Fig. 4c). These results demonstrate a close relationship between Pin1 and β-catenin levels under both physiological and pathological conditions.

Pin1 binds β-catenin phosphorylated on the Ser 246-Pro motif. To explore the molecular mechanism by which Pin1 stabilizes β-catenin, we investigated whether β-catenin is a Pin1 substrate, because β-catenin contains three potential Ser-Pro motifs. To examine the interaction between Pin1 and β-catenin *in vitro*, we used glutathione S-transferase (GST)-Pin1 pulldown experiments,
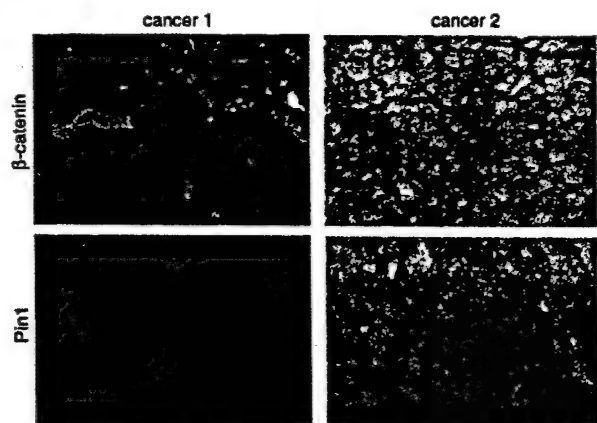
Figure 8 Correlation between Pin1 overexpression and β-catenin localization in human breast-cancer tissues. Breast-cancer tissues were stained with anti-β-catenin or anti-Pin1 antibody and revealed by staining with diaminobenzene. Left panels, a representative high-Pin1 staining with an accumulation of β-catenin in the cytoplasm and nucleus; right panels, a representative low-Pin1 staining with a localization of β-catenin at the membrane.

as described previously[32,33]. As shown in Fig. 5a, GST-Pin1, but not control GST, specifically precipitated β-catenin from both interphase and mitotic HeLa cell extracts, indicating that Pin1 binds cellular β-catenin independently of the cell cycle. Next, we examined whether Pin1 forms stable complexes with β-catenin in cells. When cells that expressed haemagglutinin-labelled Pin1 were subjected to co-immunoprecipitation experiments, Pin1 was detected in anti-β-catenin immunoprecipitates (Fig. 6a), demonstrating the interaction of these two proteins in vivo. These results demonstrate that Pin1 binds β-catenin both in vitro and in vivo.

We next examined whether the interaction between Pin1 and β-catenin depends on the phosphorylation of β-catenin at a specific Ser/Thr-Pro motif. Because the kinase(s) upstream of β-catenin remain to be determined, we produced ^{35}S-labelled β-catenin by transcription and translation in vitro, then phosphorylated it with Xenopus extracts, which have been shown to contain many protein kinases[40]. Pin1 bound ^{35}S-β-catenin only after it had been phosphorylated by interphase or mitotic extracts from Xenopus (Fig. 5b, and data not shown). Furthermore, pretreatment of phosphorylated β-catenin with calf intestinal alkaline phosphatase (CIP) completely abolished the ability of Pin1 to bind β-catenin (Fig. 5b), as demonstrated previously for the Pin1–Cdc25 or Pin1–tau interaction[32,34]. These results demonstrate that the Pin1 binding depends on the phosphorylation of β-catenin.

β-Catenin contains only three Ser-Pro motifs (Fig. 5c). To determine the Pin1-binding site in β-catenin, we constructed and expressed three different β-catenin fragments, each containing one Ser-Pro motif (Fig. 5c). When these fragments were synthesized in vitro and phosphorylated by Xenopus extracts, followed by a GST pulldown assay, only the MT2 fragment (residues 233–423), not the MT1 or the MT3 fragment, bound Pin1 (Fig. 5d). Interestingly, the MT2 fragment contained the Ser 246-Pro motif, which is surrounded by hydrophobic residues similarly to the optimal binding motif selected by Pin1, as determined by screening-orientated degenerate peptide libraries[30]. To confirm the Pin1 binding site we mutated Ser 246 of β-catenin to alanine (S246A) and performed a GST pulldown assay after incubation with Xenopus extracts. As expected, in contrast to wild-type β-catenin, the S246A β-catenin mutant did not bind Pin1 protein (Fig. 5e). These results indicate that the Pin1-binding site is the phosphorylated Ser 246-Pro in the middle of the Armadillo repeats in β-catenin.

Pin1 blocks the interaction between β-catenin and APC. The Pin1-binding site (Ser 246-Pro) in β-catenin is very close to the APC binding site, on the basis of the crystal structure of β-catenin[41,42], indicating that Pin1 might affect the interaction of β-catenin with APC. To examine this possibility, cells were transfected with Pin1 or control vectors and then subjected to immunoprecipitation with antibodies against APC or β-catenin. As shown previously[7–9], β-catenin was detected in anti-APC immunoprecipitates and APC was detected in anti-β-catenin immunoprecipitates in control-vector-transfected cells, confirming the interaction in vivo between these two proteins (Fig. 6). However, in cells overexpressing Pin1, significantly less APC was detected in anti-β-catenin immunoprecipitates (Fig. 6a). Similarly, much less β-catenin was immunoprecipitated by anti-APC antibodies (Fig. 6b). Furthermore, these differences were highly specific because the overexpression of Pin1 had no detectable effect on the binding of β-catenin to GSK-3β or of APC to the APC-binding protein EB1 (Fig. 6a, b). These results demonstrate that Pin1 specifically inhibits the interaction between β-catenin and APC.

Pin1 alters the subcellular localization of β-catenin. Recent studies have shown that APC is important in controlling the localization of β-catenin by exporting it from the nucleus to the cytoplasm[20–22,43]. If Pin1 inhibits the binding of β-catenin to APC, Pin1 might affect the subcellular localization of β-catenin. We therefore investigated the subcellular localization of β-catenin. As shown previously[20,44–46], β-catenin was observed along the inner portion of the cell membrane in the cells transfected with green fluorescent protein (GFP) vector (Fig. 7a). However, in GFP–Pin1-transfected cells, β-catenin was detected mainly in the nucleus and the peri-nuclear portion (Fig. 7a, b). In contrast, the subcellular localization of β-catenin was unaffected by the Pin1 mutants containing a mutation either in the WW domain (Pin1^{W34A}) or in the PPIase domain (Pin1^{K63A}) (Fig. 7a, b). These results indicate that both the binding and isomerization activities of Pin1 are required to increase the nuclear fraction of β-catenin.

To confirm this Pin1-induced accumulation of nuclear β-catenin, we fractionated cells into cytoplasmic and nuclear fractions and subjected them to immunoblotting with anti-β-catenin antibodies. In comparison with vector-transfected cells, the concentration of β-catenin was slightly decreased in the cytoplasmic fraction but significantly increased in the nuclear fraction in Pin1-overexpressing cells (Fig. 7c). Semi-quantification revealed that the nuclear/cytoplasmic ratio of β-catenin was increased almost threefold in cells overexpressing Pin1 (Fig. 7d). Thus, both an immunocytochemistry analysis and a cell fractionation experiment confirmed that Pin1 facilitates the nuclear localization of β-catenin, which is consistent with its abilities to disrupt the interaction between β-catenin and APC and to induce target genes downstream of that encoding β-catenin.

To determine whether the mutation of Ser 246 affects the ability of Pin1 to affect the subcellular localization of β-catenin, we cotransfected HeLa cells with Pin1 and wild-type or S246A mutant β-catenin, and then examined the subcellular localization of β-catenin. Because the overexpression of exogenous β-catenin causes its spontaneous nuclear translocation and cell death[46–48], we transfected cells with a small amount of red-fluorescent-protein (RFP)-conjugated β-catenin DNA. Under these conditions, RFP–β-catenin displayed a dotted-type aggregation in cytoplasm (Fig. 7e), as reported previously[46]. Importantly, β-catenin was stabilized and accumulated in the nucleus when co-transfected with GFP–Pin1, but not control GFP (Fig.7e, f). In contrast, Pin1 had no effect on the localization of the S246A mutant β-catenin (Fig.7e, f), which is consistent with the findings that this mutant was not a Pin1 substrate (Fig. 5e). These results indicate that the Ser 246-Pro motif of β-catenin is crucial for Pin1 to affect the subcellular localization of β-catenin.

Correlation between nuclear localization of β-catenin and overexpression of Pin1 in human breast-cancer tissues. To examine further whether Pin1 expression was correlated with the subcellular

Table 2 Summary of immunohistochemical analysis in 40 breast cancer tissues

	Pin1 staining		
	Low level	High level	
β-catenin staining	(n=11)	(n=29)	Total
Cytoplasm/nucleus	2 (5.0%)	23 (57.5%)	25 (62.5%)
Membrane only	9 (22.5%)	6(15.0%)	15 (37.5%)
Total	11 (27.5%)	29 (72.5%)	40 (100.0%)

The level of Pin 1 expression and localization of β-catenin were determined in 40 surgical specimens of breast cancer, as shown in fig. 8. The correlation was analysed using a Spearman rank correlation test P < 0.01

localization of β-catenin *in vivo*, we determined both the expression of Pin1 and the subcellular localization of β-catenin in 40 primary human breast-tumour tissues by immunohistochemical staining. As shown previously (ref. 27 and Fig. 4c), Pin1 is overexpressed to various degrees in breast-cancer tissues. Interestingly, β-catenin accumulated in the nuclear/cytoplasmic fraction in tumour tissues containing high levels of Pin1, whereas the localization of β-catenin was primarily at membranes in tumour tissues containing low levels of Pin1 (Fig. 8, Table 2). In 40 tumour tissues examined there was a significant correlation between Pin1 expression and the subcellular localization of β-catenin, as determined by the Spearman rank correlation test (P < 0.01). These results further support the notion that Pin1 is important in the regulation of β-catenin and strengthen the significance of Pin1 overexpression in the activation of β-catenin in human breast cancer.

Discussion

We have recently found that Pin1 is drastically overexpressed in several human cancer tissues, including breast and prostate cancers, and that Pin1 binds phosphorylated c-Jun and increases its ability to activate the cyclin D1 promoter via the activator protein-1 (AP-1) site[27]. These findings indicate that overexpression of Pin1 might contribute to oncogenesis via the modulation of gene expression. To examine this possibility further, we performed gene expression profiling with the differential display method. Interestingly, four of twelve upregulated genes identified are target genes downstream of that encoding β-catenin, which have been confirmed by northern and western analyses. Furthermore, Pin1 activates cyclin D1 and TOPFLASH promoters via the TCF sites. Moreover, the overexpression or depletion of Pin1 levels in cell lines significantly alters the levels of β-catenin by affecting its protein stability. The significance of this observation *in vitro* is substantiated by the findings that upregulation of Pin1 in breast cancer is strongly correlated with β-catenin levels in the tumours, whereas β-catenin levels were markedly decreased in tissues in Pin1 knockout mice. These results demonstrate that Pin1 regulates the stabilization of β-catenin under both physiological and pathological conditions.

The identification of the gene encoding cyclin D1 as one of the Pin1-induced genes in the differential display screen has confirmed our early findings that Pin1 is correlated with the overexpression of cyclin D1 in human breast cancer and activates the cyclin D1 promoter *in vitro*[27]. Pin1 can activate the cyclin D1 promoter via the AP-1 site in collaboration with the Ras signalling pathway[27]. Our present studies have further demonstrated that, as Pin1 concentrations increase, Pin1 can also activate the cyclin D1 promoter via the TCF sites through the activation of the β-catenin signalling pathway. These results indicate that Pin1 might regulate the cyclin D1 promoter via different pathways, on the basis of the gene reporter assays. This is consistent with the findings that Pin1 levels are correlated

with cyclin D1 levels in breast cancer[27]. Recently, we also found that mice lacking Pin1 demonstrate phenotypes remarkably similar to those in cyclin-D1-deficent mice (Y.-C.L., A.R., H. K. Huang, P. J. Lu, R. Bronson, F. Fujimori, T. Uchida, T. Hunter and K.P.L., unpublished results). It has been shown that high β-catenin activity is significantly correlated with cyclin D1 expression and poor prognosis, and is a strong and independent prognostic factor for human breast cancer[24]. Our findings that Pin1 is correlated with β-catenin in breast-cancer tissues indicate that Pin1 might be a potential prognostic marker.

Pin1-catalysed prolyl isomerization can induce a conformational change in proteins and thereby affects protein activity, protein dephosphorylation and/or protein–protein interactions[28–30,32–35]. Given that β-catenin contains three Ser-Pro motifs, it might be a Pin1 substrate. Indeed, Pin1 binds to β-catenin *in vivo* and *in vitro*, but only after it has been phosphorylated. The binding site has been further mapped to the pSer 246-Pro motif located at the centre of Armadillo repeats; the surrounding sequence is consistent with the Pin1 binding specificity[30]. Importantly, Pin1 selectively blocks the interaction between β-catenin and APC. Furthermore, Pin1 increases an accumulation of β-catenin in the nucleus and activates the transcription of its target genes. These results indicate that Pin1 not only binds phosphorylated β-catenin and inhibits its interaction with APC, but also decreases β-catenin turnover and increases its nuclear translocation, resulting in the upregulation of target genes downstream of that encoding β-catenin.

Recent studies on the crystal structure of the β-catenin complexes and the role of APC in a nuclear–cytoplasmic shuttling might provide an explanation of why Pin1 affects the interaction between β-catenin and APC and the accumulation of β-catenin in the nucleus. APC is a nuclear–cytoplasmic shuttling protein that can export nuclear β-catenin to the cytoplasm for degradation[20,22,43]. Interestingly, in contrast to two other Ser-Pro motifs that are buried in the helices in β-catenin, the Pin1-binding Ser 246-Pro motif is located at an exposed loop region between the two helices at the third Armadillo repeat (Supplementary Information, Fig. S1a)[41,42]. Importantly, this Ser 246-Pro motif is next to the binding site for APC, and mutations of Phe[253] and Phe[293] in β-catenin completely abolish their ability to bind APC (Supplementary Information, Fig. S1b)[41,42]. We therefore propose that Pin1 would bind and isomerize the pSer 246-Pro peptide bond in β-catenin, which would affect its ability to bind APC, thereby regulating the turnover and subcellular localization of β-catenin (see Supplementary Information, Fig. S1a, b). Consistent with this notion is the observation that disruption of the ability of Pin1 either to bind or to isomerize the pSer/Thr-Pro motifs abolishes the ability of Pin1 to induce the translocation of β-catenin to the nucleus and to activate the β-catenin-dependent transcription. Thus, our results indicate that Pin1-dependent prolyl isomerization might be a novel mechanism for regulating the β-catenin and APC interaction.

In summary, our results show that overexpression of Pin1 contributes to the upregulation of β-catenin in tumours such as breast cancer, where β-catenin is upregulated in the absence of a mutation in APC or β-catenin. Because the inhibition of the enzymatic activity of Pin1 triggers tumour cells to enter apoptosis, inhibition of upregulated Pin1 might offer a novel anti-cancer strategy. □

Methods

articles

Expression System (Clontech) in accordance with the manufacturer's protocol. Cells were collected at 24 h after induction and total RNA was extracted with TRIzol reagent (Gibco BRL). Fifty micrograms of total RNA was used for constructing the cDNA library for differential display screening as described previously[37,38], with minor modifications. cDNA fragments with two different adapters on both sides were amplified by PCR in the presence of [^{32}P]dCTP with adapter-specific primer sets. The amplified cDNAs were separated on a 6% polyacrylamide gel with urea and detected by autoradiography with X-ray film exposed overnight. By displaying about 10,000 cDNA fragments from Pin1-overexpressing breast-cancer MCF-7 cells and control MCF-7 cells, 48 obviously different bands were recovered and re-amplified by PCR with the same primer set and cloned into pUC118 vector. Recombinant plasmid DNAs were sequenced with Big Dye terminator kits (PE Applied Biosystems, Branchburg, New Jersey) with an ABI 377XL automated sequencer (PE Applied Biosystems).

Northern blot analysis.
Eight micrograms of total RNA was separated on a 1.2% agarose gel containing 0.66 M formaldehyde and transferred to a Hybond-N$^+$ membrane (Amersham) in accordance with the manufacturer's protocol. The filters were baked at 80 °C for 2 h. The cDNAs were labelled with [^{32}P]dCTP by using a Megaprime DNA-labelling System (Amersham Pharmacia). Membrane filters were hybridized with labelled probes in buffer (40% deionized formamide, 4 × SSC, 10% dextran sulphate, 1 × Denhardt's solution, 40 μg ml^{-1} salmon-sperm DNA, 0.1% SDS, 20 mM Tris-HCl pH 7.5) at 42 °C for 16 h. The filters were washed twice at room temperature for 15 min each, and once for 30 min at 56 °C with 2 × SSC containing 0.1% SDS, then exposed to films.

GST pulldown assay, immunoprecipitation and immunoblotting analyses.
Cells were arrested at the G1/S phase or the mitotic phase, as described previously[32], and β-catenin and its mutants were translated in vitro with the TNT coupled transcription/translation kit (Promega) in the presence of 8 μCi [^{35}S]methionine. They were then incubated in Xenopus extracts, as described[29]. Cell lysates, or proteins translated in vitro, were incubated with 20 μl agarose beads containing GST-Pin1 or GST at 4 °C for 2 h, as described previously[32,38]. The precipitated proteins were washed with wash buffer containing 1% Triton X-100 and subjected to SDS-PAGE.

For immunoprecipitation, cells were harvested at 24 h after transfection and lysed with Nonidet P40 lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 μg ml^{-1} leupeptin, 1.0 μg ml^{-1} pepstatin, 0.2 mM PMSF). Cell lysates were incubated for 1 h with Protein A/G-Sepharose/mouse IgG complexes. The supernatant fraction was recovered and immunoprecipitated with 2 μg anti-APC antibody (Ab-5; Oncogene Research) or anti-β-catenin antibody (Transduction Laboratories) and 30 μl Protein A/G-Sepharose. After being washed three times with lysis buffer, pellets were resuspended in 2 × Laemmli sample buffer and then analysed by SDS-PAGE. Membranes were immunoblotted with anti-β-catenin antibody, anti-GSK-3β antibody (Transduction Laboratories), anti-APC (Ab-1), anti-lamin B or anti-EB1 antibody (Oncogene Research).

Pulse-chase analysis.
Cells were grown in 60-mm dishes to 60% confluence in normal growth medium. After 24 h of transfection, cells were washed twice with HEPES-buffered saline solution (HBSS) and pulse-labelled for 1 h in 1 ml methionine- and glutamine-free minimal essential medium (Gibco BRL) supplemented with 4 mM glutamine, 10% dialysed fetal calf serum and 100 μCi [^{35}S]methionine. Labelled cells were washed twice with HBSS and rinsed with normal growth medium. Cells were harvested at various time points and subjected to immunoprecipitation with β-catenin, followed by SDS-PAGE.

Gene reporter assay.
Approximately 60%-confluent cells were transfected in triplicate in 12-well dishes with Superfect (Qiagen). Gene reporter gene assays were performed with the Dual-Luciferase reporter assay system (Promega) at 24–30 h after transfection, as described previously[26]. pRL-TK (Promega) was used as an internal control for transfection efficiency. All results are expressed as means ± s.d. for independent triplicate cultures.

Immunostaining and cell fractionation experiments.
Cells were transfected with the indicated plasmids (1 μg of GFP vector, GFP-Pin1, GFP-Pin1^{W34A} or GFP-Pin1^{K63A} in Fig. 7a, b; 0.2 μg of RFP-β-catenin or RFP-β-catenin^{S246A} with 1 μg of GFP vector or GFP-Pin1 in Fig. 7e,f) and were fixed with 3.7% buffered formaldehyde for 5 min and stained for β-catenin protein with anti-β-catenin antibody. Staining with antibodies was performed as described previously[32,38]. Nuclei were revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining for immunofluorescence microscopy. To isolate nuclear and cytoplasmic fractions, transfected cells were washed twice with cold PBS and lysed with hypotonic buffer (HEPES pH 8.0, 10 mM MgCl$_2$, 1 mM dithiothreitol, 0.1 mM EDTA). After incubation for 10 min at 4 °C, cells were treated with 0.5% Nonidet P-40 and centrifuged at 1,000 r.p.m. for 10 min. After recovery of supernatants representing cytoplasmic components, the pellets comprising nuclear extracts were washed once with hypotonic buffer and centrifuged at 1,500 r.p.m. for 5 min and lysed with 2 × Laemmli sample buffer.

Immunohistochemistry.
A breast-cancer array was purchased from Immunogex. Paraffin was removed from slides with xylene; slides were then hydrated with 100% and 75% ethanol and washed with water. The antigen recapture procedure was performed by boiling in a microwave oven for 10 min in 1 × antigen retrieval citra (Biogene). Slides were treated with PBS containing 5% goat serum and 0.1% Triton X-100 for blocking, and then with anti-Pin1 antibody or anti-β-catenin antibody at 4 °C in a humidified chamber for 12 h. After being washed with PBS, slides were incubated with biotinylated secondary antibody for 2 h. Immunohistochemical analysis was performed with a Vectastain ABC kit and DAB staining solution (Vector Laboratories, Burlingame, California).

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PIN1 Is an E2F Target Gene Essential for *Neu/Ras*-Induced Transformation of Mammary Epithelial Cells

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Oncogenes *Neu/HER2/ErbB2* and *Ras* can induce mammary tumorigenesis via upregulation of cyclin D1. One major regulatory mechanism in these oncogenic signaling pathways is phosphorylation of serines or threonines preceding proline (pSer/Thr-Pro). Interestingly, the pSer/Thr-Pro motifs in proteins exist in two completely distinct *cis* and *trans* conformations, whose conversion is catalyzed specifically by the essential prolyl isomerase Pin1. By isomerizing pSer/Thr-Pro bonds, Pin1 can regulate the conformation and function of certain phosphorylated proteins. We have previously shown that Pin1 is overexpressed in breast tumors and positively regulates cyclin D1 by transcriptional activation and posttranslational stabilization. Moreover, in Pin1 knockout mice, mammary epithelial cells fail to undergo massive proliferation during pregnancy, as is the case in cyclin D1 null mice. These results indicate that Pin1 is upregulated in breast cancer and may be involved in mammary tumors. However, the mechanism of Pin1 overexpression in cancer and its significance in cell transformation remain largely unknown. Here we demonstrate that *PIN1* expression is mediated by the transcription factor E2F and enhanced by c-*Neu* and Ha-*Ras* via E2F. Furthermore, overexpression of Pin1 not only confers transforming properties on mammary epithelial cells but also enhances the transformed phenotypes of *Neu/Ras*-transformed mammary epithelial cells. In contrast, inhibition of Pin1 suppresses *Neu*- and *Ras*-induced transformed phenotypes, which can be fully rescued by overexpression of a constitutively active cyclin D1 mutant that is refractory to the Pin1 inhibition. Thus, Pin1 is an E2F target gene that is essential for the *Neu/Ras*-induced transformation of mammary epithelial cells through activation of cyclin D1.

Phosphorylation of proteins on serine/threonine residues preceding proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of cell proliferation and transformation (6, 18, 22, 31). For example, oncogenic *Neu/Ras* signaling has shown to lead to activation of various Pro-directed protein kinases, which eventually enhance transcription of the cyclin D1 gene via multiple transcription factors, including E2F, c-*jun*/AP-1, and β-catenin/T-cell factor (TCF) (1, 3, 17, 26, 28, 47). In addition to transcriptional activation, cyclin D1 is regulated by posttranslational modifications. Phosphorylation of cyclin D1 on the Thr286-Pro site by glycogen synthase kinase 3β (GSK-3β) enhances its nuclear export and subsequent degradation (2, 9, 10).

Cyclin D1 has been shown to play a pivotal role in the development of cancer, especially breast cancer. Overexpression of cyclin D1 is found in 50% of patients with breast cancer (5, 15). Importantly, overexpression of cyclin D1, especially the mutant cyclin D1^{T286A}, can transform fibroblasts (2, 20). In contrast, inhibition of cyclin D1 expression causes growth arrest in tumor cells (4, 11, 26, 45). Furthermore, transgenic overexpression of cyclin D1 in the mouse mammary gland leads to mammary hyperplasia and eventually adenocarcinomas (55). More importantly, disruption of the cyclin D1 gene in mice completely suppresses the ability of Ha-*Ras* or c-*Neu/HER2* to induce tumor development in the mammary gland

(60). These results indicate that cyclin D1 is an essential downstream target for mammary tumorigenesis induced by Ha-*Ras* or c-*Neu* and that a major mechanism in these oncogenic processes is phosphorylation of pSer/Thr-Pro motifs.

Interestingly, the pSer/Thr-Pro motifs in proteins exist in two completely distinct *cis* and *trans* conformations, whose conversion is catalyzed specifically by the essential prolyl isomerase Pin1 (30, 34, 43, 63). By isomerizing specific pSer/Thr-Pro bonds, Pin1 has been shown to catalytically induce conformational changes in proteins following phosphorylation, thereby having profound effects on their catalytic activity, dephosphorylation, protein-protein interactions, subcellular location, and/or turnover (21, 29, 32, 44, 46, 52, 58, 59, 62). Thus, phosphorylation-dependent prolyl isomerization is a critical regulatory mechanism in phosphorylation signaling (31).

Significantly, we have previously shown that Pin1 is strongly overexpressed in many human malignancies, such as breast cancer, and that its expression closely correlates with the tumor grade and cyclin D1 expression level in tumors (44, 58). Importantly, upregulation of Pin1 has been shown to elevate cyclin D1 gene expression by activating the c-*jun*/AP-1 and β-catenin/TCF transcription factors (44, 58). Furthermore, Pin1 can bind directly to the phosphorylated Thr286-Pro motif in cyclin D1 and stabilize nuclear cyclin D1 protein by inhibiting its export into the cytoplasm, where it is normally degraded by ubiquitin-mediated proteolysis (29). Moreover, deletion of the *PIN1* gene in the mouse results in reduction of cyclin D1 levels in many tissues as well as causes many phenotypes resembling cyclin D1 null phenotypes (13, 48), including the failure of the breast epithelial compartment to undergo the

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massive proliferative changes associated with pregnancy (29). These results indicate that overexpressed Pin1 in breast cancer can positively regulate the function of cyclin D1 at the transcriptional level and by posttranslational stabilization. However, the mechanism of Pin1 overexpression in cancer and its significance in oncogenesis remain largely unknown.

The aim of this study was to further define the molecular mechanism(s) governing Pin1 expression and to investigate the role of Pin1 in the transformation of mammary epithelial cells. We demonstrate that Pin1 expression is regulated by the transcription factor E2F and is enhanced by oncogenic *Neu/Ras* signaling via E2F activation. More importantly, overexpression of Pin1 not only leads to moderate cell transformation in mammary epithelial cells but also enhances the transformed phenotypes of *Neu/Ras*-transfected mammary epithelial cells. In contrast, inhibition of Pin1 suppresses the *Neu*- and *Ras*-induced transformed phenotypes, which can be completely rescued by overexpression of a constitutively active cyclin D1 mutant that is refractory to Pin1 inhibition. These results indicate that Pin1 is a downstream target of oncogenic *Neu/Ras* signaling and plays an essential role in mammary tumorigenesis through activation of cyclin D1.

MATERIALS AND METHODS

Cloning the human *PIN1* genomic sequence and plasmid constructions. A human placenta genomic DNA library was screened with a 200-bp fragment of the human *PIN1* cDNA encoding the first exon. We screened 10^6 plaques and obtained three positive clones which had a 15-kb genomic fragment containing exon 1 of the *PIN1* gene. Selected clones were sequenced with the Big Dye terminator kits (PE Applied Biosystems, Branchburg, N.J.) with an ABI 377XL automated sequencer (PE Applied Biosystems). About 2.3 kb of the human *PIN1* promoter sequence were amplified by PCR and cloned into the pGL3-Basic vector (Promega) to create a *PIN1* promoter-luciferase construct. The GenBank accession number of the *PIN1* promoter sequence is AF501321. Several deletion mutants were created by PCR as described previously (23). Site-directed mutants were generated with a site-directed PCR mutation kit (Stratagene) according to the manufacturer's protocol.

Cell culture. Parent MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–F-12 medium supplemented with 2% horse serum, 10 μg of insulin per ml, 1 ng of cholera toxin per ml, 100 μg of hydrocortisone per ml, and 10 ng of human epidermal growth factor (Clonetics) per ml. All other cell types used in this study were maintained in DMEM supplemented with 10% fetal bovine serum or other serum conditions, as indicated below.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed as described previously (40, 41). Double-stranded oligonucleotides corresponding to the three putative E2F recognition sites in the 5'-flanking regions of the *PIN1* gene and specific mutants of these sites are listed below, with putative E2F binding sites underlined and mutations indicated in boldface type: Site A-wild-type, 5'-CGGGAGTTT<u>TTTGGCGC</u>TCGCTAAAGG-3'; Site A-mutant, 5'-CGGGAGTTT<u>TTTT**GAAG**C</u>TCGCTAAAGG-3'; Site B-wild t:ype, 5'TGCGGCGACGC<u>GCGCCAAG</u>AAGGGGT-3'; Site B-mutant, 5'-TGCGG CGACGC<u>GCGTCAAG</u>AAGGGGT-3'; Site C-wild-type, 5'-GGAGGATGGA <u>GGAGCCAAAT</u>TTAAGCAT-3'; and Site C-mutant, 5'-GGAGGATGGAG<u>G ATCCAAAT</u>TTAAGCAT-3'.

In competition assays, these double-stranded oligonucleotides were used as competitors at a 10- or 100-fold molar excess. A consensus E2F site from the adenovirus E2 promoter was used as a probe (41). Electrophoretic mobility shift assay was performed with gel shift assay systems (Promega). Recombinant glutathione *S*-transferase (GST)–E2F1 was incubated with the radiolabeled probe in binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 50 ng of poly(dI-dC) per ml, 4% glycerol] containing end-labeled DNA fragments at 25°C for 20 min. Samples were resolved on a 5% polyacrylamide native gel in 0.5× Tris-borate-EDTA. Each gel was dried and then subjected to autoradiography for 3 h.

Gene reporter assay. At ≈60% confluency, MCF-7 or HeLa cells were transfected in triplicate with luciferase reporter constructs with FuGENE 6 (Roche Diagnostics). Gene reporter assays were performed with the dual-luciferase

reporter assay system (Promega) at 24 to 36 h after transfection as described previously (44, 58). pRL-TK or pRL-CMV (Promega) was used as an internal control for transfection efficiency. All results are expressed as the mean ± standard deviation (SD) from independent triplicate cultures.

Real-time RT-PCR. Total RNA was isolated with Trizol reagent (Gibco-BRL), and single-stranded cDNA was synthesized with Superscript (Gibco-BRL). Real-time reverse transcription (RT)-PCR was performed with an ABI 7700 sequence detector system (Applied Biosystems) as described previously (58). Briefly, 50 ng of cDNA was used in duplicate per PCR run with specific primer sets for human *PIN1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data were normalized to GAPDH as an internal control according to the manufacturer's instructions.

Cyclin D1-associated kinase assay. Cells were lysed with NP-40 lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na₃VO₄, 0.5 μg of leupeptin per ml, 1.0 μg of pepstatin per ml, 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates were immunoprecipitated with protein A-agarose beads precoated with the cyclin D1 antibody DCS-11 (NeoMarkers, Fremont, Calif.), followed by the in vitro kinase assay as described previously (27).

Chromatin immunoprecipitation. The chromatin immunoprecipitation method was as described previously (57). Briefly, 5×10^6 cells were fixed by addition of formaldehyde to the tissue culture medium (final concentration, 1%). Isolated chromatin was sonicated to an average length of 0.5 to 1 kb and treated with 1 μg of mouse anti-E2F-1 antibody (Transduction Laboratories) or control mouse immunoglobulin G (IgG) for 16 h at 4°C. The complexes were immunoprecipitated with 30 μl of protein A beads and washed with immunoprecipitation buffer (100 mM Tris-HCl [pH 9.0], 500 mM LiCl₂, 1% NP-40, 1% deoxycholate). After elution and reversal of cross-links, DNA was isolated and analyzed by PCR. PCR products were visualized on a 2% agarose gel with CyberGreen.

Cell transformation assays. Transformation assays were performed as previously described (8, 16). MCF-10/*Neu/Ras* cells (10^5 per 60-mm-diameter dish) were transfected with 1 μg of pIRES-puro/GFP, pIRES-puro/GFP-Pin1, or pIRES-puro-GFP/dnPin1 by FuGENE (Roche Diagnostics). After 24 h, transfected cells were selected with puromycin (1.3 μg/ml) for 36 to 48 h. Cells were then trypsinized and passed into 100-mm-diameter dishes. The medium was changed twice weekly for 3 weeks. For colony counting, cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% acetic acid for 10 min, and stained with 0.4% crystal violet in 10% ethanol for 10 min. The dishes were rinsed, inverted, and dried at room temperature. Soft agar assays were performed in 6-cm plates with a 3-ml basal layer of 0.5% agar in 10% fetal bovine serum. A total of 5,000 to 50,000 cells in 0.3% top agar were plated in each plate in triplicate as described previously (8, 16). After 2 to 3 weeks, positive colonies (0.2-mm diameter) were counted, and the transformation efficiency was determined.

Three-dimensional Matrigel assay. Three-dimensional Matrigel assays were performed as described before (37, 42). Cells were resuspended in assay medium (DMEM–F-12 supplemented with 2% horse serum, 10 μg of insulin per ml, 1 ng of cholera toxin per ml, 100 μg of hydrocortisone per ml, and 10 ng of human epidermal growth factor per ml) at a concentration of 8×10^4 cells/ml. Eight chambered RS glass slides (Nalgene) were coated with 35 μl of Matrigel per well and left to solidify for 20 min. Then 200 μl of cell suspension was mixed 1:1 with assay medium containing 4% Matrigel and plated on each chamber. Assay medium was replaced every 4 days. After 15 days, cells were fixed with 10% methanol–acetone and stained with anti-E-cadherin antibody (Transduction Laboratories) and TOPRO-3 (Molecular Probes), followed by confocal microscopy.

RESULTS

The *PIN1* promoter is regulated by transcription factor E2F. Although Pin1 has been shown to be overexpessed in many tumors such as breast carcinoma (45, 59), the molecular mechanism of this overexpression remains unknown. We therefore decided to examine the transcriptional regulation of *PIN1* expression. As a first step to identifying the *PIN1* promoter sequence, we screened a human genomic DNA library and isolated three positive clones (Fig. 1A). A 2.3-kb fragment upstream of exon 1 of the *PIN1* gene was subcloned for further analysis. This promoter sequence has neither TATA nor CAAT boxes but has two putative GC boxes and three putative E2F binding sites, named sites A, B, and C (Fig. 1A).

A

ccctgcaaaaaataaaagaggctgggttagctttggacatctgtgggaaacaggtgggaagagggaggat

ggag**gagccaaa**tttaagcatcgggtacttaggtgctcatctccaaggcaactgcattgctatggcaaca
    E2F-C -550

gcagaggcagcaccgccccgcccaagtacctcctggaccgccgttacctctgcaccagccagactcttcc

gcatgggagggctgggggcttgcggacctccgggaacagctcaggcaggcgggagttgctgcggcattag

ccaatccataagctctatcccgcctgggtgggctctgcggcgacgc**gcgccaag**aaggggtcgggagttt
                               E2F-B -305

tttggcgctcgctaaagggcgttgagaagggcgggcccgcgtcgcttcggaccactcaggagccgaggag
E2F-A -281

agcgag**tgggcggggc**caggctgaggctggccg**ggggcggggc**gcagggataacgtgggagggtccgccc
       Sp1 -204   Sp1 -177

agcctttcttctacccaatagaaggccagaaagccaatccggaccgttagggagcccaatgggcgtcgcc

gccaggccccgttgcagagcgcgtctagccaataggcagcggcggcgggcgggcgcgggcgacaggcggc

gcagctgag**GCGGAGCAGGCGCTGCGGCAGGAGGGAAGATGGCGGACGAGGAGAAGCTGCCGCCCGGCTG**
      +1

B

C

FIG. 1. E2F binds the *PIN1* promoter. (A) Human *PIN1* promoter sequence. The nucleotide sequence of the human *PIN1* gene that includes the 5'-flanking region and first exon is listed. Putative binding sites for transcription factors are underlined. The ATG translation initiation codon is in the first exon typed in boldface. (B) Electrophoretic mobility shift assays were performed with recombinant E2F1 protein and end-labeled double-stranded oligonucleotides (oligo) corresponding to the *PIN1* promoter sequence containing either wild-type (wt) or mutant (mt) E2F binding sites. A consensus E2F site from the adenovirus E2 promoter was used as a competitor (comp.) in a 100× molar excess of labeled probe. (C) Competitive activity of *PIN1* promoter sequences for E2F binding. Labeled oligonucleotides corresponding to E2F binding sites from the adenovirus E2 promoter were incubated with recombinant E2F1 protein in the presence or absence of unlabeled *PIN1* promoter sequences. Three different oligonucleotides corresponding to E2F binding sites in the *PIN1* promoter (sites A to C) were used as competitors. Wild-type oligonucleotides were mixed at a 10- or 100-fold molar excess and mutants were mixed at a 100-fold molar excess of labeled probe.

The fact that the E2F/Rb pathway is deregulated in many cancers suggested a possible role for E2F in overexpression of Pin1 in cancer cells. To examine whether E2F binds the *PIN1* promoter, we first synthesized double-stranded oligonucleotides corresponding to each putative E2F site and conducted electrophoretic mobility shift assays with recombinant E2F1 protein. Recombinant E2F1 bound all three E2F probes, and the binding was completely abolished by point mutations introduced into each putative E2F site (Fig. 1B). Furthermore, a competition assay with nonlabeled oligonucleotides revealed that the wild-type but not the mutant oligonucleotides competed efficiently for E2F1 binding to the Pin1 E2F sites (Fig. 1B). Moreover, all three putative E2F sequences also efficiently competed with the adenovirus E2 promoter sequence, a well-characterized E2F site (38), for E2F1 binding (Fig. 1C). However, no competition was detected with any of three mu-

tant E2F-binding sequences (Fig. 1C). These results indicate that all three putative E2F-binding sequences have the ability to bind E2F1 in vitro.

To examine whether E2F affects *PIN1* promoter activity and whether any of the putative E2F sites are functional in vivo, we inserted the 2.3-kb 5'-flanking region of the *PIN1* gene into a basic luciferase expression vector (pGL3-Basic), resulting in −2300LUC, and performed gene reporter assays. Indeed, E2F1 effectively activated the *PIN1* promoter in a dose-dependent manner (Fig. 2A). Furthermore, two other E2F proteins, E2F2 and E2F3, also potently activated the *PIN1* promoter (Fig. 2B). These results indicate that E2F proteins can activate the *PIN1* promoter in cells.

To determine the importance of three putative E2F-binding sites in the *PIN1* promoter, several 5' deletional and site-directed mutants were generated (Fig. 1E). Compared to the
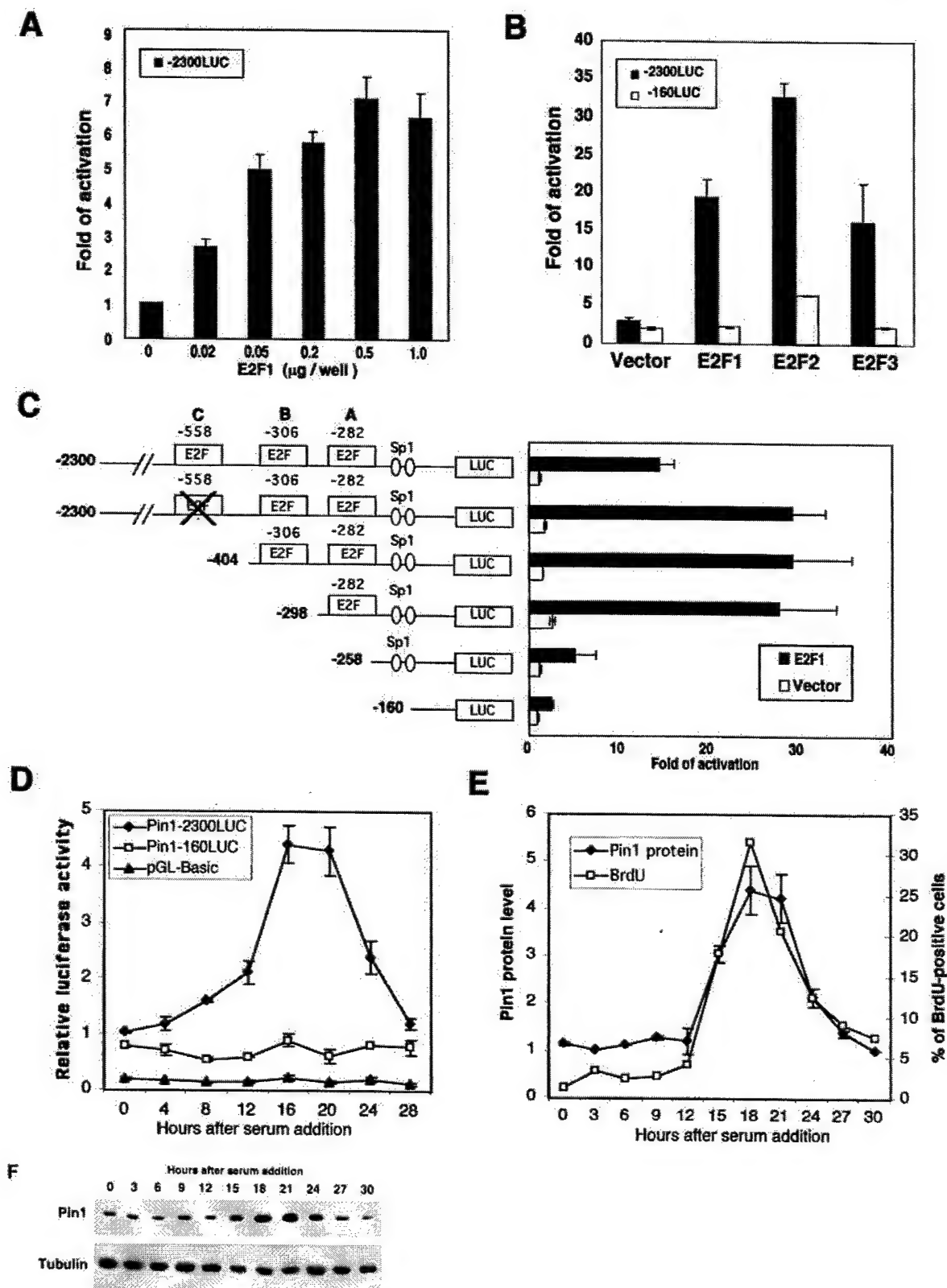
FIG. 2. Activation of the *PIN1* promoter by E2F. (A) E2F1 activates *PIN1* promoter activity in a dose-dependent manner. MCF-7 cells were transfected with the *PIN1* promoter-luciferase construct (−2300LUC) and E2F-1 expression vector. Cells were harvested at 36 h after transfection and subjected to a gene reporter assay. (B) E2F family proteins enhance *PIN1* promoter activity. Cells were cotransfected with vectors expressing E2F1, E2F2, or E2F3 together with the −2300LUC or −160LUC reporter construct. (C) Mapping of the *PIN1* promoter region responsible for transcriptional activation by E2F. A series of 5′ deletion and site-directed mutants were transfected into HeLa cells together with the E2F1 expression vector or a control vector. (D) Cell growth-dependent regulation of *PIN1* gene expression in normal fibroblasts. MEFs were transfected

wild-type *PIN1* promoter (−2300bp), the mutation or deletion of the two distant E2F-binding sites (sites C and B, located at −557 to −550 and −312 to −305, respectively) did not have much effect on *PIN1* promoter activity in response to E2F1 (Fig. 2C). However, deletion of the proximal E2F-binding site (site A, located at −288 to −281) strongly diminished the induction of the *PIN1* promoter by E2F1 (Fig. 2C). Similar results were also observed in the induction of the *PIN1* promoter by either E2F2 or E2F3 (data not shown). These results indicate that the proximal E2F-binding site is the most important regulatory site for the *PIN1* promoter by E2F.

In addition, the *PIN1* promoter has two GC boxes, which are potential recognition sites for the transcription factor Sp1 (Fig. 1A). Since several reports have shown a possible functional interaction of Sp1 with E2F (25), we deleted these two Sp1-binding sites (−160LUC). Deletion of the Sp1 sites slightly reduced *PIN1* promoter activity, suggesting that they may also contribute to full induction of *PIN1* by E2F (Fig. 1E). These gene reporter assays demonstrate that E2F1 can regulate the promoter activity of the *PIN1* gene.

To determine whether other E2F family members affect *PIN1* promoter activity, expression vectors encoding E2F1 to -3 were cotransfected with *PIN1* promoter reporter constructs. Figure 2B shows that E2F family proteins, especially E2F2, potentiated *PIN1* promoter activity through E2F-binding sites. However, the −160-base construct, in which the three E2F and Sp1 binding sites were deleted (−160LUC), was not responsive to the ectopic expression of E2F proteins. These results indicate that in addition to E2F1, E2F2 and E2F3 can also activate the *PIN1* promoter.

Many E2F downstream target genes are related to cell cycle progression and DNA synthesis and are regulated in a cell growth-dependent manner in normal cells, especially when E2F-binding sites are proximal to the transcription initiation site (14, 39–41). To examine whether *PIN1* expression is dependent on cell growth, we measured *PIN1* promoter activity and Pin1 protein levels in normal mouse embryo fibroblasts (MEFs) at various time points following cell cycle reentry. MEFs were synchronized at the G_0 phase by serum starvation and then released to enter the cell cycle by the addition of serum. Although the activity of Pin1−160LUC and control vectors did not respond to serum addition, *PIN1* promoter activity was upregulated 16 to 20 h after serum addition (Fig. 2D). This correlated with an increase in Pin1 protein levels in the same cells within the same time frame following serum addition (Fig. 2E and F). Moreover, increased *PIN1* promoter activity and Pin1 protein levels correlated well with DNA synthesis, as assayed by bromodeoxyuridine (BrdU) incorporation (Fig. 2F). These results indicate that *PIN1* gene expression is regulated in a cell cycle-dependent manner in normal cells, further supporting the role of E2F in regulating *PIN1* expression in cells.

Binding of E2F to the *PIN1* promoter in vivo correlates with *PIN1* expression in breast cell lines. We have previously shown that in cancer cells, Pin1 protein levels are constant throughout the cell cycle and remain at higher levels than those in normal or nontransformed cell lines. The above results suggested that constitutive deregulation of the Rb/E2F pathway may contribute to Pin1 overexpression in cancer cells. To address this possibility, we first examined whether E2F binds the *PIN1* promoter sequence in several nontransformed and transformed breast epithelial cell lines by chromatin immunoprecipitation analysis with an anti-E2F1 antibody. Although each input sample had a similar amount of the *PIN1* promoter sequence when tested by quantitative PCR (Fig. 3A), there were dramatic difference in the amounts of *PIN1* promoter sequence that were immunoprecipitated by anti-E2F antibodies, as determined by quantitative PCR with the same primer set (Fig. 3B). Compared with normal breast epithelial 76N cells and immortalized but nontransformed MCF-10A cells, much more *PIN1* promoter sequences were coimmunoprecipitated with E2F1 in several breast cancer cell lines (Fig. 3B). These results not only confirm that E2F indeed binds the *PIN1* promoter sequence in the cell but also indicate that the amounts of E2F protein bound on the promoter vary among different breast epithelial cell lines.

We next determined whether E2F binding to the *PIN1* promoter is correlated with *PIN1* expression levels by measuring *PIN1* mRNA and protein levels in these cell lines by real-time PCR and immunoblotting analyses, respectively. Both *PIN1* mRNA and protein levels were much higher in all transformed cell lines compared with those in normal primary 76N cells and immortalized but nontransformed MCF-10A cells (Fig. 3C and D). Furthermore, there was a good correlation between the amounts of E2F bound on the *PIN1* promoter and levels of *PIN1* mRNA and protein in all breast epithelial cells examined (Fig. 3B to D). Taken together, the above results demonstrate that the transcription factor E2F plays an important role in the regulation of *PIN1* expression in breast cell lines.

***Ras* and *Neu* enhance *PIN1* expression via E2F.** Oncogenic *Neu* and *Ras* signaling has been shown to enhance E2F transcriptional activity in breast cancer cells (12, 27, 39). Given that the *PIN1* promoter is activated by E2F and significantly elevated in breast cancer cells, this *Neu* and *Ras* signaling might enhance *PIN1* promoter activity via E2F activation. To examine this possibility, we first examined whether Ha-*Ras* and c-*Neu* activates the *PIN1* promoter. A wild-type *PIN1* promoter construct and a mutant construct containing point mutations in the three putative E2F-binding sites (Fig. 4A) were used in the assay. Like E2F, Ha-*Ras* and c-*Neu* transactivated the *PIN1* promoter by ≈10-fold and ≈5-fold, respectively (Fig. 4B). However, point mutations at the three E2F-binding sites completely abolished the ability of Ha-*Ras* or *Neu* to transactivate the *PIN1* promoter (Fig. 4B). These results suggest that

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with the indicated luciferase reporter constructs and induced to enter quiescence by serum starvation (0.05% serum) for 48 h. The medium was then supplemented with serum (15%), allowing cells to reenter the cell cycle as a synchronous population. Cells were harvested at various time points and subjected to gene reporter assays. (E and F) MEFs were synchronized by serum starvation as for panel D. Prior to harvesting, cells were treated with BrdU for 30 min. Cells were collected at indicated time points and subjected to immunoblotting analysis with anti-Pin1 antibody or flow cytometory analysis with anti-BrdU antibody. Band intensities in Pin1 protein levels (F) were quantified by using NIH-Image and graphed with the results from the BrdU study (E).
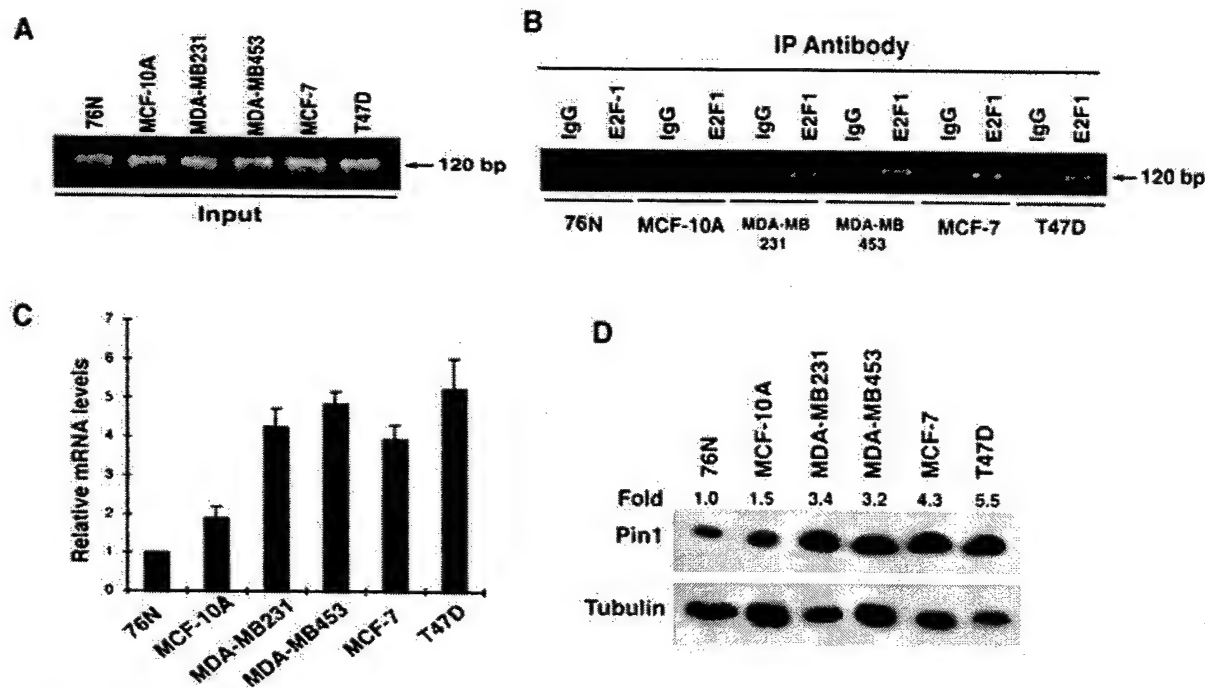
FIG. 3. E2F binding to the *PIN1* promoter sequence in vivo correlates with Pin1 expression level in breast cancer cell lines. (A and B) Levels of E2F binding to the *PIN1* promoter in different breast cell lines. Cross-linked chromatin from exponentially growing breast cancer cell lines was incubated with either antibodies against E2F1 or control IgG. Immunoprecipitates from each sample were analyzed by PCR with primers specific for the *PIN1* promoter sequence (B). As an input control, total input chromatin was analyzed by PCR with the same primer set (A). (C and D) Levels of *PIN1* mRNA and protein in different breast cell lines. mRNAs were isolated from the cell types indicated, and *PIN1* mRNA was quantified by real-time RT-PCR analysis and normalized to GAPDH mRNA (C). *PIN1* levels were determined by subjecting cell lysates to immunoblotting analysis with a monoclonal anti-Pin1 antibody (D), Numbers above the gel image indicate the induction (fold) of Pin1 protein level normalized to α-tubulin.

Ras and *Neu* transactivate the *PIN1* promoter through its E2F sites.

To further confirm this result, we used an E2F1 mutant (E2F1E132) which has been well shown to inhibit the function of the endogenous E2F proteins in a dominant-negative fashion (24). Cotransfection of *Ras* and wild-type E2F highly increased *PIN1* promoter activity (Fig. 4C). However, cotransfection with the dominant-negative E2F mutant significantly decreased the ability of *Ras* to transactivate the *PIN1* promoter (Fig. 4C). Similar phenomena were also observed in *Neu*-induced *PIN1* promoter activation (data not shown). These gene reporter assays suggest that *Neu/Ras* signaling transactivates the *PIN1* promoter via E2F.

To ensure that *Ras* and *Neu* can increase *PIN1* expression in cells, we first examined the effects of exogenous Ha-*Ras* or *Neu* expression on *PIN1* mRNA and protein levels in mammary epithelial cells. Indeed, both Ha-*Ras* and *Neu* significantly increased *PIN1* mRNA levels in MCF-10A cells, as determined by quantitative RT-PCR (Fig. 4D), as well as Pin1 protein levels, as determined by immunoblotting analysis in MCF7 cells (Fig. 4E). Furthermore, cotransfection with E2F further increased Pin1 protein levels induced by Ha-*Ras* or *Neu* (Fig. 4E). Given that Ha-*Ras* and c-*Neu* can increase Pin1 expression in cells, we also examined whether transgenic overexpression of mouse mammary tumor virus (MMTV)–c-*Neu* or MMTV–Ha-*Ras* elevates Pin1 expression in mouse breast tissues. Interestingly, breast tissues obtained from both MMTV–

c-*Neu* and MMTV–Ha-*Ras* transgenic mice contained much higher Pin1 protein levels than control breast tissues (Fig. 4F). Taken together, these results indicate that *Ras* and *Neu* enhance *PIN1* expression both in vitro and in vivo.

Overexpression of *PIN1* confers transformed properties on mammary epithelial cells. We have previously demonstrated that *PIN1* expression is highly elevated in human breast cancer tissues and plays a pivotal role in the regulation of cyclin D1 function (29, 44, 58). Interestingly, cyclin D1 is also an essential mediator in the development of breast cancer induced by oncogenic *Neu* and *Ras* (26, 60). Given that *PIN1* is a downstream target of oncogenic *Neu/Ras* signaling, a critical question is whether overexpression of *PIN1* has any effect on the cell transformation of mammary epithelial cells.

To address this question, we stably transfected GFP-Pin1 and control GFP into MCF-10A cells, a spontaneously immortalized but nontransformed mammary epithelial cell line that has been widely used for cell transformation studies (7, 51). Multiple stable cell lines that had similar properties were obtained, with one GFP-expressing and two GFP-Pin1-expressing cell lines (clones 1 and 2) being further characterized. The two GFP-Pin1-expressing cell lines moderately overexpressed Pin1, about three- and sixfold higher than endogenous levels (Fig. 5A and data not shown). Consistent with our previous studies (29, 44, 58), cyclin D1 levels were elevated in these GFP-Pin1 stable clones compared with control GFP cells, with cyclin D1
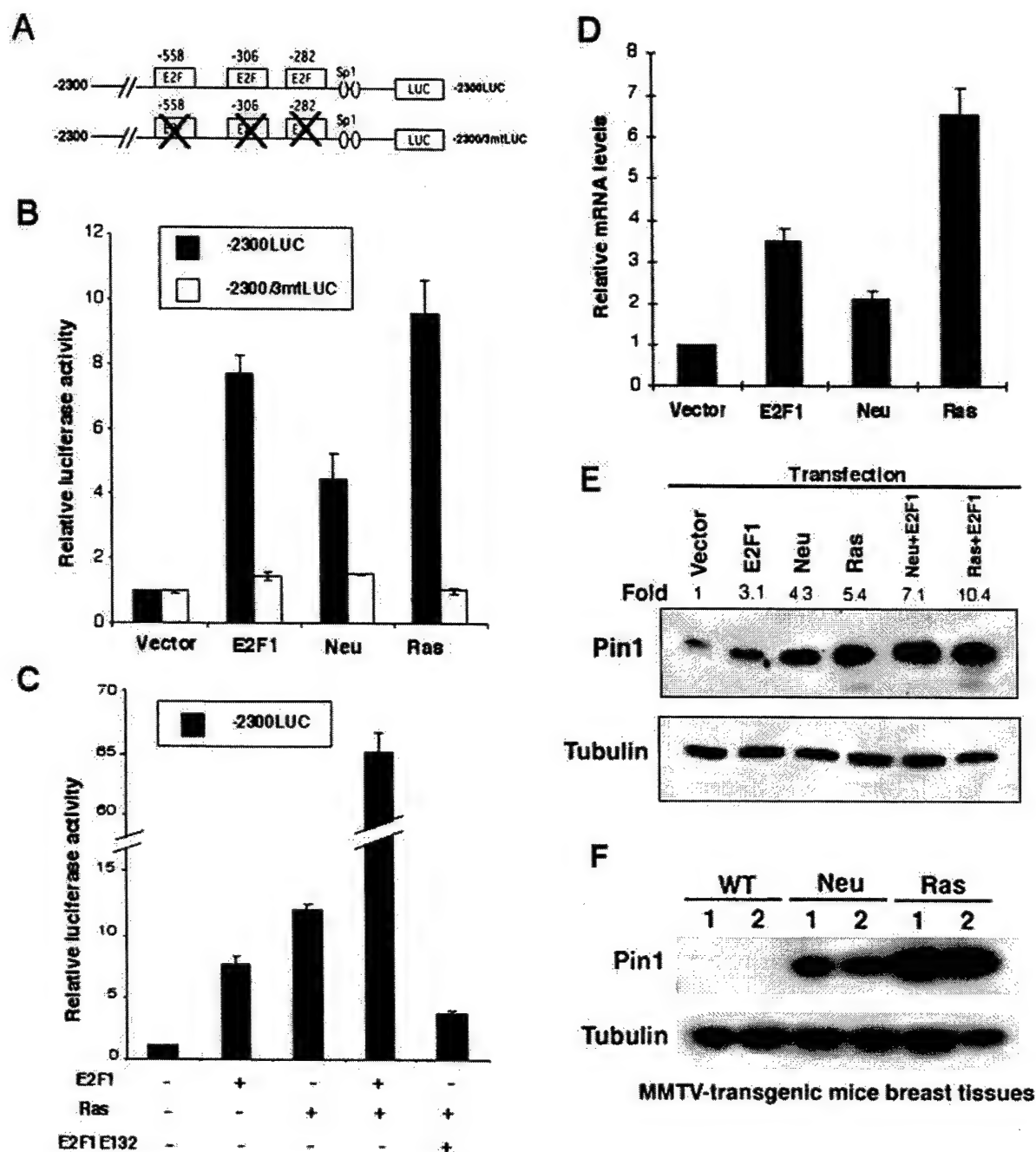
FIG. 4. *Ras* and *Neu* stimulate the *PIN1* promoter through E2F activation. (A) Schematic representation of wild-type and mutant *PIN1* promoter reporter constructs. (B) MCF-7 cells were cotransfected with a reporter construct (0.1 μg) and E2F-1, Ha-*Ras*, or *Neu* (0.5 μg), followed by gene reporter assays. (C) Dominant-negative E2F1 inhibits activation of the *PIN1* promoter by *Ras*. Gene reporter assays were performed in MCF-7 cells as shown in panel B. Wild-type E2F-1 or its dominant-negative mutant E2F1E132 was cotransfected with the *Ras* and −2300LUC reporter constructs. (D) *Neu* and *Ras* upregulate *PIN1* mRNA levels in MCF-10A cells. MCF-10A cells were transiently transfected with plasmids encoding E2F1, Ha-*Ras*, or *Neu*. For each transfection, a plasmid encoding a puromycin resistance gene (pIRES-puro) was cotransfected as a selection marker. Puromycin (1.3 μg/ml) was added to the medium 24 h after transfection. At 36 h following addition of puromycin, puromycin-resistant cells were reseeded and cultured for an additional 24 h. Total RNA was collected and subjected to real-time RT-PCR as described in Materials and Methods. (E) MCF-7 cells were transfected with the indicated expression vectors for 48 h, and cell lysates were subjected to immunoblotting analysis with anti-Pin1 and antitubulin antibodies. Numbers above the gel image indicate the fold induction of the Pin1 protein level normalized to α-tubulin. (F) *PIN1* is overexpressed in breast tissues from MMTV–c-*Neu* and MMTV–Ha-*Ras* mice. Mammary tissues from two wild-type (WT), two MMTV-*Neu* (*Neu*), and two MMTV-*Ras* (*Ras*) mice were lysed and subjected to immunoblotting analysis with anti-Pin1 and antitubulin antibodies.
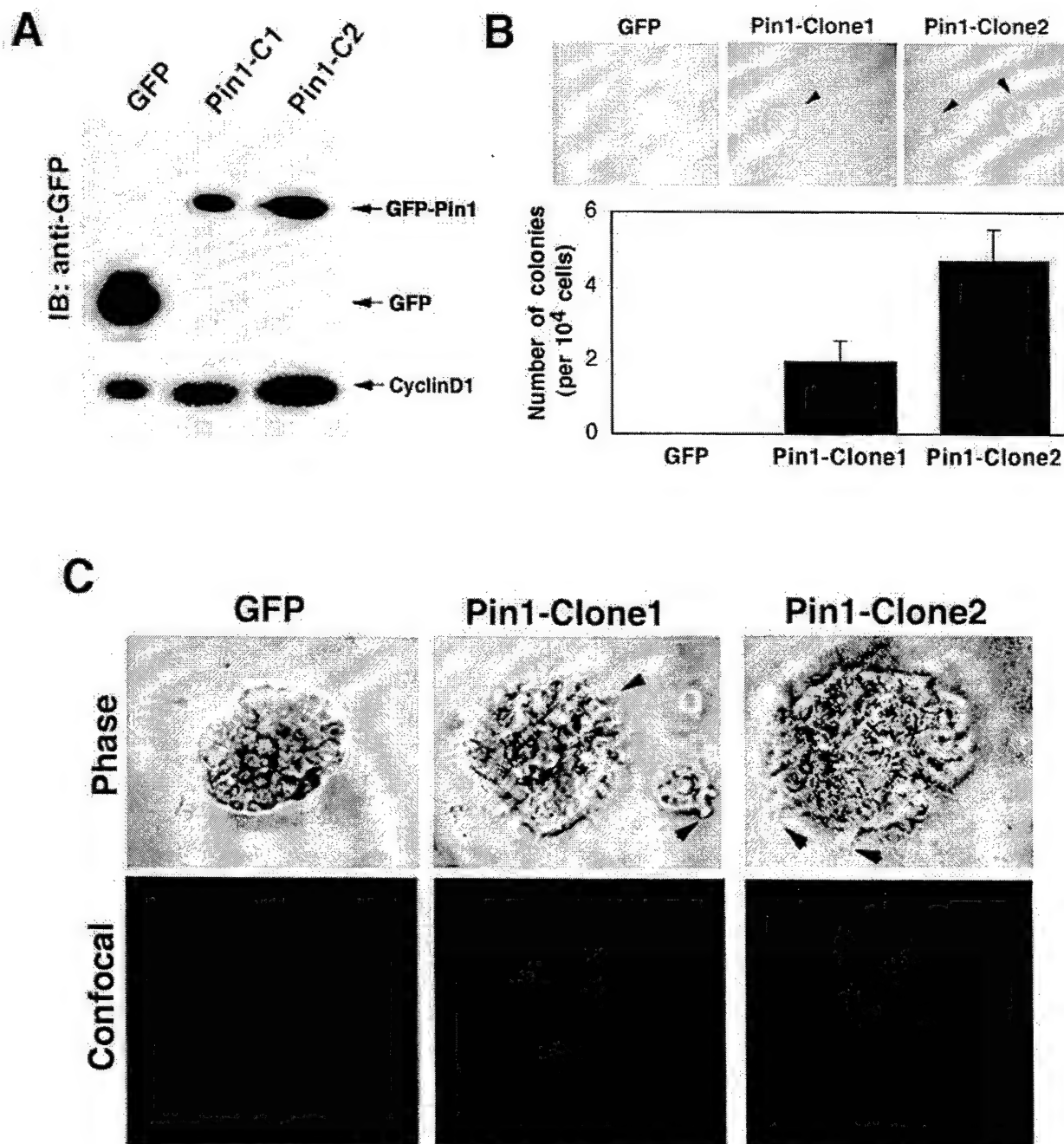
FIG. 5. *PIN1* overexpression confers a transformed phenotype on MCF-10A cells. (A) Establishment of MCF-10A cells stably expressing GFP or GFP-Pin1. Immunoblotting (IB) analysis was performed with anti-GFP and anti-cyclin D1 antibodies. (B) To measure anchorage-independent cell growth and survival, GFP- or GFP-Pin1-transfected MCF-10A cells were suspended in 0.3% soft agar for 14 days. (C) Cell lines stably expressing GFP and GFP-Pin1 were plated on Matrigel for 15 days. Phase images of an acinus at higher magnification are shown in the upper panels. The acini were stained with anti-E-cadherin antibodies and the DNA dye TOPRO-3, and confocal images though the middle of an acinus are shown in the lower panels. Arrows indicate cell surface spikes protruding into the Matrigel.

levels being correlated with exogenous Pin1 expression levels (Fig. 5A).

Although there was no detectable difference in cell morphology and growth rate on plastic plates between GFP-Pin1 and control GFP cell lines (data not shown), overexpression of GFP-Pin1 but not GFP conferred anchorage-independent cell growth in soft agar (Fig. 5B). However, the size and frequency of colonies were much less than those of *Neu/Ras*-transformed MCF-10A cells (Fig. 5B versus Fig. 6F). Moreover, like parental MCF-10A cells (7, 51), GFP-Pin1 stable cell lines were unable to survive in DMEM supplemented with10% fetal bovine serum (data not shown), while *Neu/Ras*-transformed
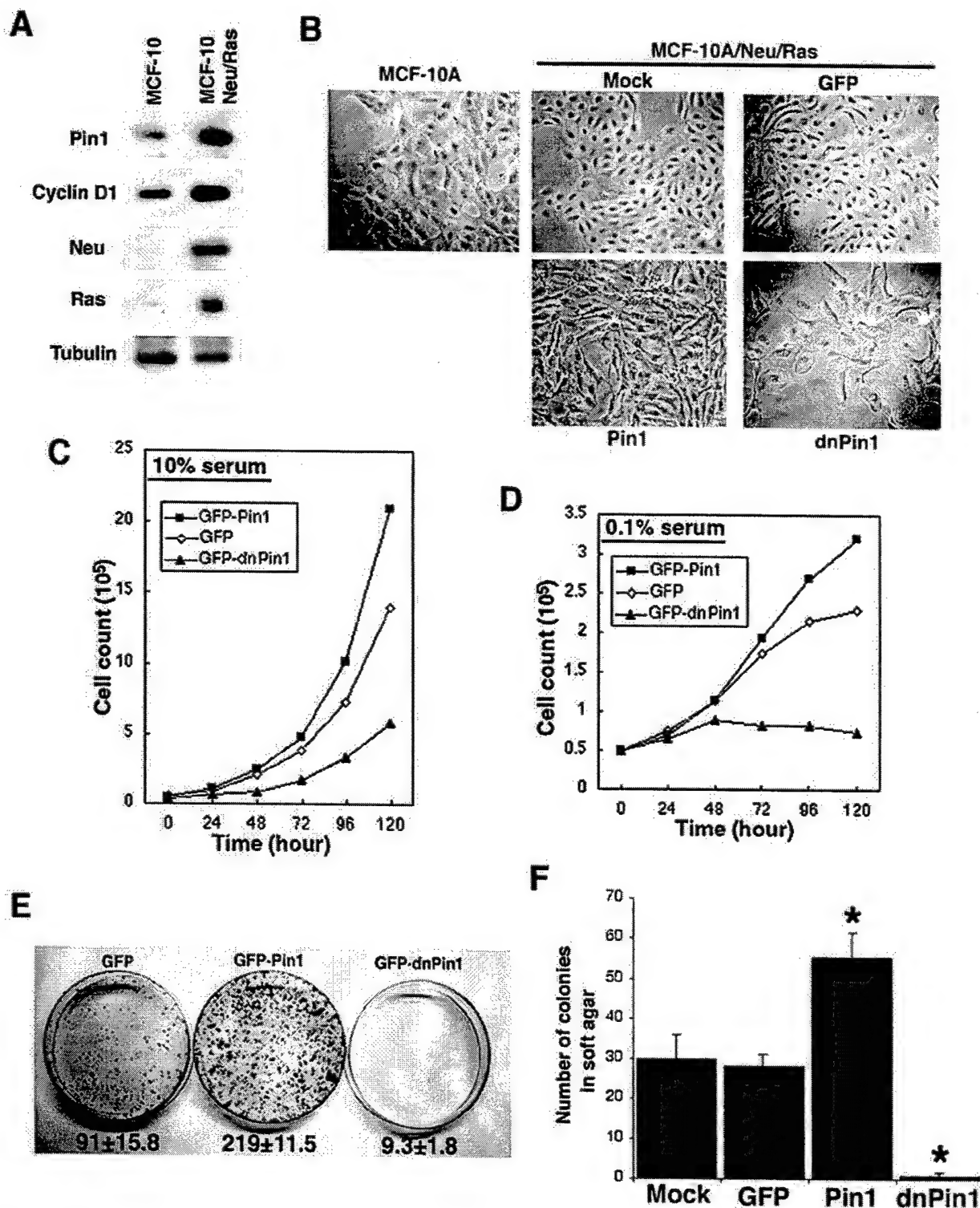
FIG. 6. *PIN1* is essential for *Neu/Ras*-induced cell transformation. (A) Establishment of stable MCF-10A cell lines expressing both *Neu* and *Ras*. Cells were cotransfected with *Neu* and *Ras* expression vectors and selected with G418. A selected clone was checked for expression of *Ras, Neu, PIN1*, and cyclin D1 by immunoblotting analysis. (B) Morphological changes in MCF-10A cells stably expressing *Neu* and *Ras* (MCF-10/*Neu/Ras*) and additional *PIN1* or dn-*PIN1*. Cells were seeded in 60-mm dishes and photographed with a phase-contrast microscope before reaching confluence. (C and D) Manipulation of Pin1 levels alters proliferation in MCF-10/*Neu/Ras* cells. Transfected cells were selected with puromycin for 48 h and reseeded in 35-mm dishes. Cells were grown in high-serum (10%) (C) or low-serum (0.1%) (D) medium and trypsinized at various time points. Viable cells were counted by the trypan blue dye exclusion method. (E) *PIN1* is necessary for *Neu/Ras*-induced focus formation. The same number of cells (10^4) transfected with either the GFP, GFP-Pin1, or GFP-dnPin1 expression vector were seeded in 10-cm plastic dishes after selection with puromycin. After 14 days, cells were fixed and stained with crystal violet. Numbers below plates indicate colony numbers (mean ± SD) in three independent experiments. (F) Cells were plated in 0.3% soft agar and cultured for 2 weeks. After 14 days, colony formation was scored microscopically. MCF-10/*Neu/Ras* cells expressing GFP-Pin1 demonstrated significant increases in anchorage-independent growth, whereas dn-Pin1 overexpression significantly blocked the growth of MCF-10/*Neu/Ras* cells. ∗, $P < 0.01$, t test.

MCF-10A cells grew normally in this medium (Fig. 6C). These data suggest that although overexpression of Pin1 appeared to be insufficient to fully transform MCF-10A cells, it might trigger some early events of cell transformation.

To further investigate this possibility, we performed a three-dimensional cell differentiation assay with exogenous basement membrane matrix (Matrigel). This method has been well established to assess the transformed phenotype of mammary epithelial cells, especially at early stages of tumorigenesis (37, 42). We found that GFP-expressing cells formed acini with basally polarized nuclear organization, intact cell-cell junctions, and visible lumina inside, as indicated by immunostaining with antibodies against the cell-cell junction marker E-cadherin and with the DNA dye TOPRO-3, followed by confocal microscopy (Fig. 5C). These structures are known as well-differentiated acini that are usually observed in parental MCF-10A cells (37, 42), indicating that expression of GFP had no effect. However, expression of GFP-Pin1 had a dramatic effect on the morphology and organization of acinar formation. Colonies formed by GFP-Pin1-expressing cells exhibited disorders in nuclear polarity and cell arrangement without a lumen inside, disruption of basement membrane, and impairment in cell-cell junction (Fig. 5B, lower). Furthermore, GFP-Pin1 but not GFP-expressing cells had cell surface spikes protruding into the Matrigel (Fig. 5C, top). Since a lack of acinar organization is a specific event involved in progression towards malignancy (37), these results suggest that Pin1 overexpression can induce events associated with early stages of mammary tumorigenesis. However, additional events might be needed to lead to the full transformation of mammary epithelial cells.

Overexpression of *PIN1* enhances whereas inhibition of *PIN1* suppresses transformed phenotypes of mammary epithelial cells induced by *Neu* and *Ras*. It is also well established that oncogenic *Neu/Ras* signaling induces cell transformation of mammary epithelial cells via upregulation of cyclin D1 (26, 60). Given that *PIN1* is a downstream target of *Neu/Ras* signaling and regulates cyclin D1 function, we hypothesized that *PIN1* mediates *Neu/Ras* signaling thorough the activation of cyclin D1 during breast cancer formation.

To test this hypothesis, we first examined whether manipulating cellular *PIN1* function affects the transformed phenotype of mammary epithelial cells induced by *Neu* and *Ras*. To address this question, we needed to establish MCF-10A cells stably expressing c-*Neu* and Ha-*Ras* together (MCF-10/*Neu*/*Ras*), because overexpression of both *Neu* and Ha-*Ras* has been shown previously to induce a transformed phenotype mimicking the malignancy of mammary carcinomas (16). MCF-10/*Neu*/*Ras* cells exhibited elevated levels of both cyclin D1 and Pin1 compared with parental MCF-10A cells (Fig. 6A). These results are consistent with the findings that *Neu*/*Ras* signaling increases expression of cyclin D1, as shown previously (26, 60), and of Pin1, as shown above.

Morphologically, MCF-10/*Neu*/*Ras* cells demonstrated a higher nuclear/cytoplasmic ratio and multiple nucleoli, which is consistent with their higher proliferation rate, than the parental MCF-10A cells (Fig. 6B). These cells were able to grow in DMEM supplemented with a high concentration of fetal calf serum (10%) or even with low serum (0.1%) (Fig. 6C and D), as well as in soft agar (Fig. 6F). These results indicate that

MCF-10/*Neu*/*Ras* cells display various transformed phenotypes, as reported previously (16).

With this cell line, we investigated whether upregulation or downregulation of Pin1 affects the transformed phenotypes. To inhibit cellular Pin1 function, we used a Pin1 WW domain construct, which contains an Ala substitution at Ser16. This construct has been shown to inhibit endogenous Pin1 interaction with target substrates, thereby functioning as a dominant-negative *PIN1* (dn-*PIN1*) (33). Given that excessive overexpression of *PIN1* or dn-*PIN1* blocks cell cycle progression (3, 33), we used rather low concentrations of expression constructs after a series of pretests. MCF-10/*Neu*/*Ras* cells were transfected with GFP-Pin1, GFP-dnPin1, or control GFP, followed by selection with puromycin. The transfection efficiencies and expression levels of three different constructs were comparable in each set of transfectants, as confirmed by scoring GFP-fluorescent cells under a microscope or by immunoblotting analysis with anti-GFP antibodies (Fig. 7A and data not shown). Compared with parental and GFP-expressing cells, GFP-Pin1-expressing cells exhibited a higher nuclear/cytoplasmic ratio, with disorganized cell arrangements (Fig. 6B). In contrast, GFP-dnPin1-expressing cells exhibited large and vacuolar morphology with higher density of cytoplasmic speckles, similar to those of parental MCF-10A cells (Fig. 6B). These morphological changes suggest that expression of GFP-Pin1 and GFP-dnPin1 might affect the transformed phenotypes of these cells.

To examine this possibility, we next examined the cell proliferation rate in high-serum (10%) and low-serum (0.1%) medium. Compared with GFP control cells, GFP-Pin1-expressing cells grew faster, whereas dn-*PIN1*-expressing cells grew much more slowly in 10% serum medium (Fig. 6C). Furthermore, GFP-expressing control cells grew even in low serum (Fig. 6D), consistent with the fact that MCF-10/*Neu*/*Ras* transformed cells have lost the cell cycle checkpoint induced by a low concentration of growth factors, as shown previously (8, 16). More interestingly, GFP-Pin1-expressing cells continued to grow linearly even when the growth of GFP-transfected cells was retarded in low-serum medium after 48 h (Fig. 6D). In contrast, dn-*PIN1*-expressing cells could not grow under low-serum conditions (Fig. 6D). These results suggest that overexpression of Pin1 increases cell proliferation and transformed phenotypes of MCF-10/*Neu*/*Ras* cells, whereas inhibition of Pin1 reverses these phenotypes.

To further support this observation, we investigated the long-term cell proliferation and transformation properties of these cells by performing colony formation assays on plastic plates and in soft agar. Consistent with the short-term cell growth study, expression of GFP-Pin1 increased colony formation, doubling the number of colonies compared with GFP control cells, both on plastic plates and in soft agar (Fig. 6E and F). Furthermore, individual colonies were much larger (Fig. 6E). In contrast, dn-*PIN1* overexpression dramatically inhibited colony formation; these cells produced very tiny colonies on plastic plates, and colony formation in soft agar was almost completely inhibited (Fig. 6E and F). Similar inhibitory effects were also seen with inhibition of Pin1 via expression of an antisense *PIN1* construct (data not shown), which has been shown to deplete endogenous Pin1 proteins (30, 44, 48). These results indicate that overexpression of Pin1 enhances *Neu*/*Ras*-
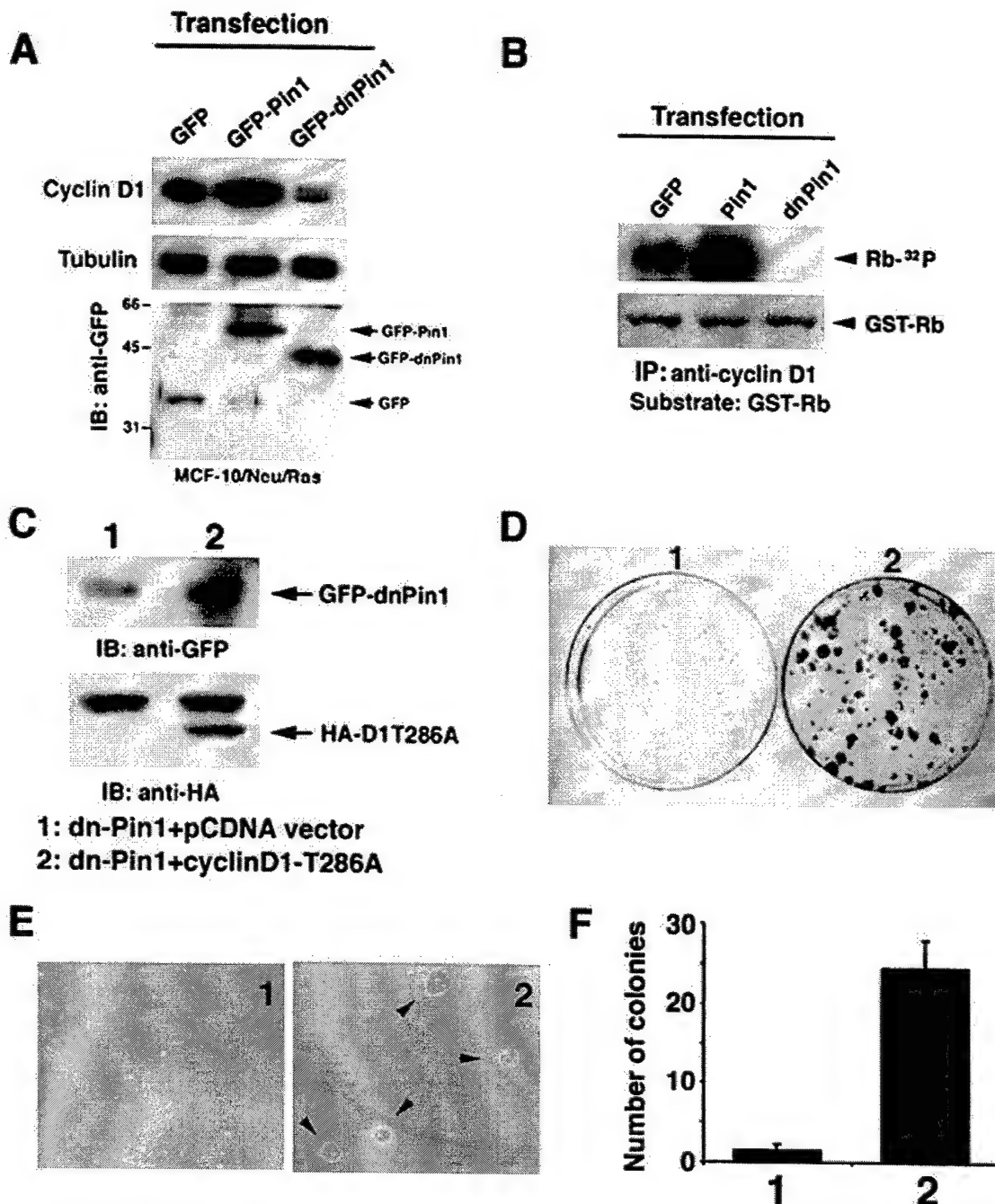
FIG. 7. Pin1 inhibition is complemented by overexpression of a constitutively active cyclin D1. (A) Pin1 is essential to maintain cyclin D1 level and activity in *Neu/Ras*-transformed MCF-10 cells. MCF-10/*Neu/Ras* cells transfected with either GFP, GFP-Pin1, or GFP-dnPin1 were lysed and immunoblotted with anti-cyclin D1, -tubulin, and -GFP antibodies. (B) The same cell lysates as in panel A were immunoprecipitated with anti-cyclin D1 antibodies, followed by the in vitro kinase assay with GST-pRB as a substrate. Phosphorylation of the GST-pRB substrate is shown in the upper panel. The lower panel shows input GST-pRB stained with Coomassie blue. (C) MCF-10/*Neu/Ras* cells were transfected with either dnPin1 and pCDNA vector or dnPin1 and the cyclin D1^{T286A} mutant (1:10 ratio) and selected with puromycin for 48 h. Cells were subjected to immunoblotting analysis with anti-HA and anti-GFP antibodies. (D) Cells were transfected as described for C and seeded on plastic plates for 3 weeks. Cells were fixed and stained with crystal violet. (E and F) Cells were transfected as described for panel C and cultured in 0.3% soft agar for 3 weeks. The number of colonies formed was scored. Representative phase pictures are shown in panel E. Colony numbers are the mean ± SD of three independent experiments (F).

induced cell proliferation and transformation, whereas the inhibition of Pin1 reverses the cell proliferation and transformed properties induced by *Neu* and *Ras*.

PIN1 affects *Neu/Ras*-induced cell transformation of mam- mary epithelial cells via cyclin D1. Cyclin D1 is an essential downstream target of *Neu/Ras*-induced mammary tumorigenesis (26, 60). Furthermore, *PIN1* positively regulates cyclin D1 function via transcriptional activation as well as posttransla-

tional stabilization (29, 44, 58). These results suggest that *PIN1* might affect *Neu/Ras*-induced cell transformation via cyclin D1. To examine this possibility, we assayed levels of cyclin D1 and its associated kinase activity. Consistent with phenotypic changes as described above, levels of cyclin D1 and its associated cyclin-dependent kinase activity were enhanced in cells expressing GFP-Pin1 compared to GFP-expressing cells (Fig. 7A and B). In contrast, both the cyclin D1 level and its kinase activity were substantially lowered by the overexpression of dn-*PIN1* (Fig. 7A and B). These results indicate that overexpression of Pin1 enhances but inhibition of Pin1 strongly inhibits cyclin D1 expression and function in *Neu/Ras*-transformed MCF-10A cells, consistent with the notion that Pin1 affects *Neu/Ras*-induced cell transformation via cyclin D1.

If this is the case, overexpression of a constitutively active cyclin D1 mutant (cyclin D1^{T286A}), which cannot bind Pin1 and is refractory to Pin1 inhibition (29), should rescue the transformed phenotypes that are inhibited by dn-*PIN1*. This experiment is also important in addressing whether suppression of transformed phenotypes by Pin1 inhibition is specifically due to inhibition of cyclin D1 or simply due to induction of cell apoptosis. We cotransfected MCF-10/*Neu/Ras* cells with GFP-dn-*PIN1* and hemagglutinin (HA)-cyclin D1^{T286A} or control vector pCDNA at a 1:10 ratio and selected transfected cells with puromycin. Immunostaining with anti-HA antibody confirmed that almost all HA-cyclin D1^{T286A}-positive cells expressed GFP-dnPin1 (data not shown), which was also confirmed by immunoblotting analysis of GFP-dnPin1 with anti-GFP antibodies and of HA-cyclin D1^{T286A} with anti-HA antibodies (Fig. 7C).

MCF-10/*Neu/Ras* cells cotransfected with dn-*PIN1* and pCDNA vector failed to form foci on plastic plates and colonies in soft agar (Fig. 7D to F), confirming that inhibition of Pin1 suppresses the transformed phenotypes induced by *Neu* and *Ras* (Fig. 6). Importantly, MCF-10/*Neu/Ras* cells expressing both dn-*PIN1* and cyclin D1^{T286A} formed many foci on plastic plates (Fig. 7D). Moreover, these cells even displayed anchorage-independent cell growth to form colonies in soft agar to the same extent, like MCF-10/*Neu/Ras* cells (Fig. 7E and F versus Fig. 6F). These results show that overexpression of a constitutively active cyclin D1 mutant can reverse the ability of Pin1 inhibition to suppress the *Neu*- and *Ras*-induced transformed phenotypes, further indicating that Pin1 affects *Neu/Ras*-induced cell transformation via cyclin D1.

DISCUSSION

In this report, we have demonstrated that Pin1 expression is regulated by the transcription factor E2F, which is enhanced by oncogenic *Neu* or *Ras*. Furthermore, overexpression of Pin1 alone is sufficient to induce normal mammary epithelial cells to display several transformed properties that have been shown to be present in the early stages of tumorigenesis. Importantly, overexpression of Pin1 enhances the transformed phenotypes of mammary epithelial cells induced by *Neu* and *Ras*. In contrast, inhibition of Pin1 suppresses the *Neu*- and *Ras*-induced transformed phenotypes, which can be fully rescued by overexpression of a constitutively active cyclin D1 mutant that is refractory to Pin1 inhibition. This is the first demonstration that *PIN1* is an E2F downstream target gene and that *PIN1*
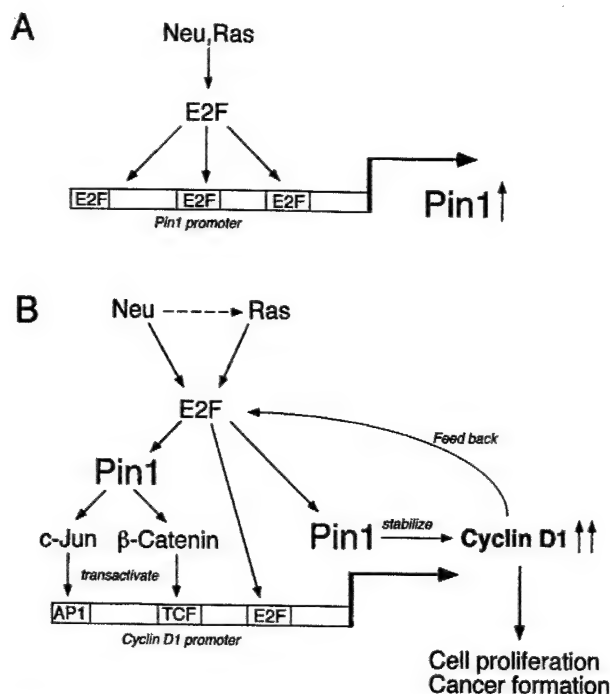
FIG. 8. Schematic models for Pin1 transcriptional regulation and its role in regulation of cyclin D1 by *Neu/Ras* signaling. (A) Oncogenic *Neu-Ras* signaling transactivates the *PIN1* promoter through E2F activity. (B) *PIN1* is a downstream target of oncogenic *Neu/Ras* signaling and is essential for *Neu/Ras*-induced cyclin D1 activation and cell transformation. *PIN1* upregulated by *Neu/Ras* signaling enhances β-catenin and c-Jun signaling to transactivate the cyclin D1 gene. Furthermore, Pin1 binds directly to cyclin D1 and stabilizes it via a posttranslational mechanism. It is possible that cyclin D1 also regulates Pin1 expression via E2F in a positive feedback loop.

plays an essential role in *Neu/Ras*-induced mammary tumorigenesis via cyclin D1.

Pin1 as an E2F downstream target gene. The following results indicate that the transcription factor E2F plays a critical role in regulation of Pin1 expression (Fig. 8A). First, E2F family proteins activated the *PIN1* promoter specifically through the E2F binding sites. Second, E2F bound the *PIN1* promoter in vitro and in vivo. Third, the levels of E2F binding to the *PIN1* promoter correlated with the levels of Pin1 expression in breast cancer cell lines. Fourth, *PIN1* gene expression in normal cells was regulated in a cell cycle-dependent manner, as is the case for other E2F target genes (14, 39–41). Finally, overexpression of E2F enhanced *PIN1* promoter activity and mRNA levels in breast cancer cells.

Interestingly, E2F1 has been found to be a good prognostic or predictive marker of breast cancer because the E2F1 index significantly correlates with histological grade, stage, and metastatic status of breast tumors (61). Similarly, Pin1 expression is correlated with the histological grade of breast cancer (58). These results indicate that deregulation of E2F may play a key role in the upregulation of Pin1 in breast cancer. Since deregulation of the Rb/E2F pathway is also found in many other cancer types and contributes to the oncogenesis of a number of human cancers (18, 35, 39, 53, 56), deregulation of the Rb/E2F pathway may also contribute to Pin1 overexpression in other

cancer cells. Further experiments are needed to examine the role of E2F in regulating Pin1 expression in other cancers.

Significance of Pin1 overexpression in cell transformation. We report here for the first time that overexpression of Pin1 can play an important role in the transformation of mammary epithelial cells. Phosphorylation of proteins on Ser/Thr-Pro is a key regulatory mechanism in controlling cell proliferation and transformation (2, 18, 22, 63). The conformation and function of many phosphorylated proteins are regulated by the phosphorylation-specific prolyl isomerase Pin1 (31). Interestingly, Pin1 is highly overexpressed in many human cancer tissues, including breast cancer cells, but its significance in cell transformation is largely unknown (44, 58). We have now found that overexpression of Pin1 conferred an anchorage-independent cell growth phenotype on normal mammary epithelial cells, although the colony size and frequency of Pin1-overexpressing cells were smaller and lower than those of *Neu/Ras*-transformed cells. In addition, Pin1-expressing cell lines failed to grow in 10% fetal bovine serum, in contrast to *Neu/Ras*-transformed cells.

These data suggest that overexpression of Pin1 might trigger some early events during cell transformation. This was further supported by a three-dimensional Matrigel assay, which has been well established to assess the transformed phenotype of mammary epithelial cells, especially at early stages of tumorigenesis (37, 42). Indeed, expression of Pin1 had a dramatic effect on the morphology and organization of acinar formation. These cells exhibited the disorder in the nuclear polarity and cell arrangement without a lumen inside, disruption of the basement membrane, and impairment in cell-cell junction. Furthermore, Pin1-expressing cells had cell surface spikes that protruded into the Matrigel. These results indicate that overexpression of Pin1 might disrupt normal differentiation in mammary epithelial cells. Since the lack of acinar organization and the presence of cell surface spikes have been suggested to be specific events in progression towards malignancy (16, 37), these results suggest that Pin1 overexpression can induce events associated with the early stages of mammary tumorigenesis.

Essential role of Pin1 for the *Neu/Ras*-induced transformation of mammary epithelial cells. Our exciting observation was that Pin1 plays an essential role in the transformation of mammary epithelial cells by *Ras* and *Neu* via activation of cyclin D1. Various studies have revealed that *Neu* or *Ras* signaling is deregulated in many breast cancers, although mutations and amplifications of these genes were rarely observed (3, 19, 50, 54). Furthermore, transgenic overexpression of MMTV–Ha-*Ras* or MMTV–c-*Neu* potently induces mammary tumors via cyclin D1. However, transgenic overexpression of MMTV-cyclin D1 has much weaker tumorigenicity (36, 49, 55). In addition, constitutive overexpression of cyclin D1 alone cannot transform MCF-10A cells, nor is it sufficient to prevent G_1 arrest induced by EGF deprivation (7). These discrepancies could be explained by the findings that cyclin D1 is regulated not only by transcriptional activation but also by posttranslational stabilization.

Phosphorylation of cyclin D1 on the Thr286-Pro site by GSK-3β enhances its nuclear export and subsequent degradation (2, 9, 10). In fact, EGF deprivation results in a rapid degradation of cyclin D1 in MCF-10A cells constitutively over-expressing cyclin D1 (7). In contrast to wild-type cyclin D1, mutant cyclin $D1^{T286A}$ is stable and functions as a constitutively active mutant which can potently transform mammary epithelial cells (2). These results suggest that both transcriptional activation and posttranslational stabilization of cyclin D1 are critical for tumor development induced by *Neu/Ras* signaling. Interestingly, we have previously shown that by binding and isomerizing specific pSer/Thr-Pro motifs, Pin1 cooperates with *Ras*-JNK-c-Jun and the wnt-β-catenin-TCF pathways to enhance cyclin D1 expression (44, 58). Furthermore Pin1 can also enhance the stability and nuclear localization of cyclin D1 by directly binding and presumably isomerizing the phosphorylated Thr286-Pro motif (29). Therefore, Pin1 positively regulates the function of cyclin D1 both at the transcriptional level and via posttranslational stabilization, resulting in the transformation of mammary epithelial cells.

Our studies have demonstrated that *Neu/Ras* signaling can activate expression of Pin1 and that overexpression of Pin1 in *Neu/Ras*-expressing mammary epithelial cells enhances their transformed phenotypes. In contrast, inhibition of Pin1 by a dominant-negative mutant or an antisense construct dramatically reduced both cell proliferation and the transformed phenotypes of these cells. Importantly, this inhibitory effect by Pin1 inhibition was rescued by overexpression of a cyclin D1-T286A mutant, which is refractory to Pin1 inhibition (29). These results suggest a model in which *Neu/Ras* signaling can activate expression of Pin1, which in turn enhances the expression and stability of cyclin D1, eventually leading to cell proliferation and transformation (Fig. 8B).

It appears that upregulation of Pin1 does not precede cyclin D1 upregulation during cell cycle reentry of normal MEFs. However, deletion of the *PIN1* gene results in a significant decrease in both cyclin D1 mRNA and protein levels in MEFs and also causes phenotypes in mice resembling those of cyclin D1 null mice (29), indicating an important role of *PIN1* in regulating cyclin D1 function in normal conditions. Therefore, it is possible that cyclin D1 regulates *PIN1* expression via E2F in a positive feedback loop (Fig. 8B). This might provide an explanation for why oncogenic *Ras* or *Neu* is more potent than cyclin D1. Therefore, *PIN1* might be a key player in modulating upregulation of cyclin D1 by *Neu* and *Ras* oncogenic signaling.

In summary, our results provide the first evidence for a requirement for appropriate regulation of *PIN1* gene expression in transformation of mammary epithelial cells induced by *Neu* and *Ras*. *PIN1* is upregulated by *Neu* and *Ras* via E2F. Furthermore, overexpression of *PIN1* not only can confer transformed properties on normal mammary epithelial cells but also can enhance the transformed phenotypes induced by *Neu* and *Ras*. Finally, inhibition of *PIN1* suppresses *Neu*- and *Ras*-induced transformed phenotypes via cyclin D1. These results indicate that overexpression of *PIN1* in human cancer cells would help promote tumor cell growth and also suggest that *PIN1* inhibitors could be useful for anticancer therapies.

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Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes

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Phosphorylation of proteins on serine/threonine residues preceding proline is a key signaling mechanism. The conformation and function of a subset of these phosphorylated proteins is regulated by the prolyl isomerase Pin1 through isomerization of phosphorylated Ser/Thr-Pro bonds. Although young Pin1$^{-/-}$ mice have been previously shown to develop normally, we show here that they displayed a range of cell-proliferative abnormalities, including decreased body weight and testicular and retinal atrophies. Furthermore, in Pin1$^{-/-}$ adult females, the breast epithelial compartment failed to undergo the massive proliferative changes associated with pregnancy. Interestingly, many of these Pin1-deficient phenotypes such as retinal hypoplasia and mammary gland impairment are also the characteristic of cyclin D1-deficient mice. Cyclin D1 levels were significantly reduced in many tissues in Pin1-deficient mice, including retina and breast epithelial cells from pregnant mice. Moreover, Pin1 directly bound to cyclin D1 phosphorylated on Thr-286–Pro increased cyclin D1 in the nucleus and stabilized cyclin D1. These results indicate that Pin1 positively regulates cyclin D1 function at the transcriptional level, as demonstrated previously, and also through posttranslational stabilization, which together explain why Pin1 loss-of-function phenotypes in the mouse resemble cyclin D1-null phenotypes. Our results provide genetic evidence for an essential role of Pin1 in maintaining cell proliferation and regulating cyclin D1 function.

Phosphorylation of proteins on serine/threonine residues preceding proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of various cellular processes, including cell division and transcription (for reviews see refs. 1–3). The pSer/Thr-Pro moiety in peptides and proteins exists in two distinct *cis* and *trans* conformations, whose conversion is catalyzed specifically by Pin1 (4, 5). Pin1 is a *cis/trans* peptidyl-prolyl isomerase that acts only on phosphorylated Ser/Thr-Pro bonds (6–8). In addition, Pin1 contains an N-terminal WW domain, which functions as a phosphorylated Ser/Thr-Pro binding module (9, 10). This phosphorylation-dependent interaction targets Pin1 to a defined subset of phosphorylated substrates facilitating conformational changes in phosphorylated proteins, thereby regulating their biological function (7, 11–20). Thus, Pin1-dependent prolyl isomerization is an essential and novel postphosphorylation regulatory mechanism.

Given its phosphorylated Ser/Thr-Pro substrate specificity, Pin1 has also been shown to be essential for maximal cell growth in different systems (4, 5). Interestingly, we have recently found that Pin1 is strikingly overexpressed in most human breast cancer tissues (21, 22). Pin1 levels are correlated with cyclin D1 mRNA and protein levels in human cancer tissues. Moreover, Pin1 can activate the cyclin D1 promoter in cell lines via binding phosphorylated c-Jun and β-catenin and increasing their transcriptional activity (21, 22). These results suggest that Pin1 may play an important role in regulation of cyclin D1 expression and also contribute to neoplastic transformation. Interestingly, disruption of cyclin D1 results in several prominent phenotypes, including retinal degeneration and mammary gland impairment (23, 24). However, disruption of the Pin1 gene in mice has been previously reported to develop normally

(25). Therefore, the genetic connection between Pin1 and cyclin D1 remains to be established. Furthermore, although turnover and subcellular localization of cyclin D1 is regulated by phosphorylation on Thr-286–Pro motif by GSK-3β (26–28), it is unknown whether it is further regulated after phosphorylation.

Here, we found a range of cell-proliferative abnormalities, including decreased body weight and testicular and retinal atrophies. Interestingly, some of these phenotypes are also characteristic of cyclin D1-deficient mutant mice. In addition, we found that Pin1 directly bound to and stabilized cyclin D1 in nucleus, indicating that Pin1 regulates stability and subcellular localization of cyclin D1, in addition to the transcriptional regulation of the cyclin D1 gene we reported previously (21, 22). This study provides direct evidence that Pin1 plays a critical role in the regulation of cyclin D1 and suggests a novel mechanism for regulating cyclin D1 function.

Materials and Methods

Immunohistochemical Analysis. For immunohistochemical analysis, both age-matched wild-type and knockout mice tissues were perfused and fixed by using Bouin's fixation solution. The immunostaining was carried out as described (13). Briefly, the fixed tissues were embedded in paraffin and sectioned at 6 μm. The dissected sections were deparaffinized in xylene, hydrated through an alcohol series from 100 to 50%. To inhibit endogenous peroxidase, sections were treated with H_2O_2. Antigen recapture was done by boiling slides in 1× antigen retrieval Citra (Biogenex Laboratories, San Ramon, CA). Primary antibody incubations were performed overnight at 4°C in a humidified chamber. Affinity-purified anti-Pin1 antibodies were as described (13), and polyclonal cyclin D1 antibodies were raised against a C-terminal peptide and purified by using the antigen peptide, as described (13). For the cyclin D1 control slides, cyclin D1 primary antibody was incubated with the excess antigenic peptide for 2 h before use. Immunohistochemical analysis and DAB staining were performed by using a Vectastain ABC kit (Vector Laboratories) as described (21, 22).

Mammary Gland Whole Mounts. To examine the development of the mammary epithelium during pregnancy, the no. 4 mammary glands of nulliparous and 1-day postpartum wild-type and Pin1$^{-/-}$ mice were dissected, spread onto a glass slide, and fixed overnight in 6:3:1 methanol:chloroform:acetic acid buffer. The

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fixed glands were washed by using 70% alcohol with several changes, then defatted with acetone once or twice for 2 h each. The glands were stained overnight in 0.2% carmine red (Sigma) and 0.5% AlK(SO4)$_2$, dehydrated in graded ethanol solutions, followed by clearing in toluene and methyl salicylate. Photos were taken by using a dissecting microscope (23, 29).

Glutathione *S*-Transferase (GST)-Pull Down Assay. HeLa cells were transiently transfected either hemagglutinin (HA)-tagged cyclin D1-wild type or HA-tagged cyclin D1^{T286A} mutant for 24 h. Cell lysates were incubated with 20 μl of agarose beads containing GST-Pin1 or GST for 2 h at 4°C as described (22). The precipitated proteins were washed three times in wash buffer containing 1% Triton X-100 and subjected to SDS/PAGE, as described (11).

Pulse–Chase Analysis. Primary embryonic fibroblasts were prepared from 14.5-day embryos. MEF cells were grown in 60-mm dishes to 60% confluence in normal growth medium. Cells were transfected with HA-cyclin D1 and CDK4 by using Effectene (Qiagen, Chatsworth, CA). After 16 h of transfection, cells were washed twice with Hanks' balanced salt solution and pulse-labeled for 40 min in 1 ml of methionine- and glutamine-free MEM (GIBCO/BRL) supplemented with 4 mM glutamine/ 10% dialyzed FCS/100 μCi of [^{35}S]methionine, as described (22, 26). Labeled cells were washed twice with Hanks' balanced salt solution and rinsed with normal growth medium. Cells were harvested at various time points and subjected to immunoprecipitation with the 12CA5 monoclonal antibody.

Expression and Localization of Cyclin D1 in MFE Cells. Exponentially growing Pin1$^{-/-}$ or wild-type MEF cells were placed on glass plates and stained with anti-cyclin D1 polyclonal antibody (Santa Cruz Biotechnology). Total DNA was visualized with 4′,6-iamidino-2-phenylindole (DAPI) staining followed by the fluorescent microscopic analysis as described (22, 28). For localization experiment, Pin1$^{-/-}$ MEF cells transfected with either GFP or GFP-Pin1 were arrested in G$_0$ by serum deprivation and contact inhibition, and then cells were fixed with 3.7% formaldehyde at 18 h after serum addition. Subcellular localization of cyclin D1 was determined by staining with cyclin D1 antibodies and DAPI as described above.

Preparation of Nuclear Extracts. A nuclear fraction was prepared as described (22). Briefly, cells were washed with PBS and resuspended in hypotonic solution (10 mM Hepes, pH 7.8/10 mM KCl/2 mM MgCl$_2$/1 mM DTT/0.1 mM EDTA supplemented with protease inhibitors). After 10 min at 4°C, Nonidet P-40 was added to 1%; the cells were centrifuged for 1 min, and the nuclear pellet was briefly washed with hypotonic buffer and resuspended in SDS sample buffer.

Results

Phenotype of Pin1$^{-/-}$ Mice. By careful analysis of Pin1$^{-/-}$ mice generated previously (25), we found that they remarkably displayed a range of abnormalities, including decreased body weight, testicular atrophy, retinal degeneration, and mammary gland proliferative impairment. From birth to ≈3 months, Pin1$^{-/-}$ mice were indistinguishable from their wild-type controls, but adult Pin1$^{-/-}$ mice appeared smaller than wild-type controls (Fig. 1 *A* and *B*). At the age of ≈7 months, the body weight of Pin1$^{-/-}$ mice was significantly smaller for both male and female animals, with the average weight being only 71% of that of Pin1$^{+/+}$ mice (Fig. 1 *A* and *B*).

Effects on Seminiferous Tubules. Although Pin1$^{-/-}$ female and male mice were fertile, the fact that the success rate of homozygous cross breeding was much lower or took much longer than that of wild-type or heterozygous mice led us to suspect that Pin1$^{-/-}$ mice might develop fertility problems. To determine whether Pin1
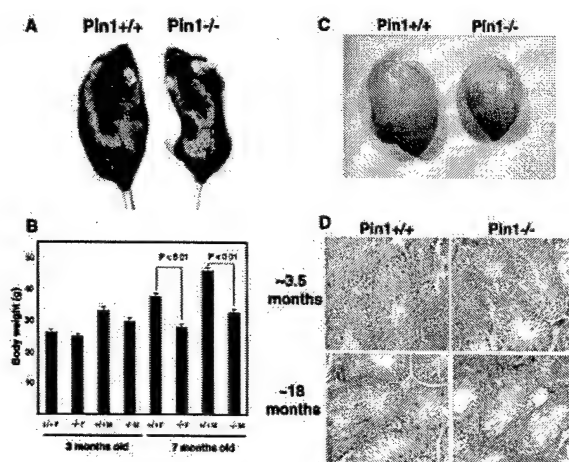
Fig. 1. Reduced body weight and testicular atrophy in Pin1$^{-/-}$ mice. (*A* and *B*) Reduced body weight. Representative adult wild-type mouse (*Left*) and Pin1$^{-/-}$ mouse (*Right*) are shown in *A*. A comparison of body weight of 10 wild-type and Pin1$^{-/-}$ mutant male and female mice at ~3.5 and ~7 months is shown in *B*. (*C* and *D*) Testicular atrophy, as indicated by representative testis from wild-type or Pin1$^{-/-}$ mouse at ~3.5 months old (*C*) or by histopathological comparison (*D*). Testicular sections obtained from ~3.5- and ~16-month-old mice were stained with hematoxylin and eosin.

affects sexual maturation, we first examined the development of the ovary and testis. Ovarian tissues lacking Pin1 appeared to have normal morphology and histology. On the other hand, all autopsied Pin1$^{-/-}$ males had testicular atrophy. By 3–5 months of age, the average weight of six Pin1$^{-/-}$ testes was only 56% of that of wild-type controls (Fig. 1*C*, data not shown). Moreover, this striking weight difference of testis was not due to the smaller body weight because Pin1$^{-/-}$ males were not significantly smaller than the wild-type controls at this age (Fig. 1*B*). Histological examination revealed that the seminiferous-tubule degeneration could be detected at the age of ≈3.5 months old (Fig. 1*D*). By ≈15 months and even more pronounced by 18 months old, the seminiferous tubules in Pin1$^{-/-}$ mice degenerated, with basically no mature sperm in the lumen, whereas age-matched wild-type mice exhibited healthy seminiferous tubules (Fig. 1*D*). These observations suggest that Pin1 may play a critical maintenance role in adult spermatogenesis, and its absence of Pin1 may result in defects in spermatogonial cell division, meiosis, or sperm maturation.

Effects on Retinal Tissue. As part of our analysis of the phenotypes of Pin1$^{-/-}$ mice, we found that Pin1$^{-/-}$ mice exhibited retinal degeneration at ≈16 months of age. The thickness of retinal layers in Pin1$^{-/-}$ mice was strikingly reduced (Fig. 2*A*). To quantify the retinal degeneration, we determined the number of nuclear cell layers in six retinas. Wild-type mice contained an average of ≈7-cell diameter thickness in the inner and 13-cell diameter thickness in the outer nuclear layers, whereas Pin1$^{-/-}$ mice contained only ≈4-cell diameter thickness in the inner and 7-cell diameter thickness in the outer nuclear layers (Fig. 2*C*). Given the retinal degenerative phenotype in old Pin1$^{-/-}$ mice, we also examined the eyes at 1 day after delivery and at 4–6 months to examine whether younger Pin1$^{-/-}$ mice have a similar abnormality. Although the retinal layers in Pin1$^{-/-}$ mice were similar to those of Pin1$^{+/+}$ mice at birth (data not shown), about 50% of Pin1$^{-/-}$ eyes displayed mild retinal degradation by 4–6 months of age (Fig. 2*B*). These results indicate that the Pin1$^{-/-}$ retina undergoes degeneration and that, like the testicular atrophy, this is also age-dependent (Fig. 1 *C* and *D*),
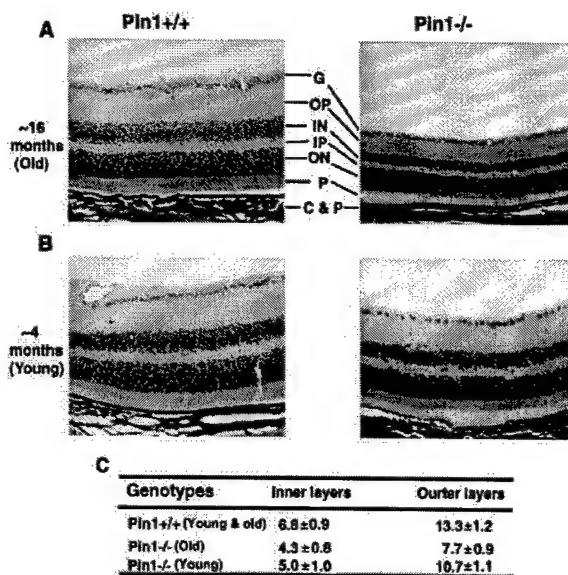
Fig. 2. Retinal atrophy in Pin1$^{-/-}$ mice. (A and B) Histopathological examination of retinas. Sections through age-matched wild-type (*Left*) and Pin1$^{-/-}$ (*Right*) retinas at ~16-month-old (A) or ~4-month-old (B) mice were stained with hematoxylin and eosin. The layers indicated are as follows: G, surface ganglion cell layers; OP, outer plexiform layer; ON, outer nuclear layer; IN, inner nuclear layer; P, photoreceptor cell layer; C&P, choroid and pigment cells. (C) Comparison of the number of the nuclear layers. The numbers of the inner and outer nuclear layers were counted from six age-matched wild-type (*Left*) and Pin1$^{-/-}$ (*Right*) retinas, with the average and standard deviations being presented. Because there is no difference between young and old animals, the results are combined.

suggesting that Pin1 may play an important role in survival or cell proliferation in the retina.

Effects on Mammary Gland. One of the most striking ailments found in cyclin D1 mutant mice was that the mammary gland fails to undergo full lobuloalveolar development during pregnancy. These observations prompted us to examine whether Pin1-deficient mice also display impaired mammary gland associated with pregnancy. As shown in Fig. 3 A and B, the adult epithelial ducts from both Pin1$^{+/+}$ and Pin1$^{-/-}$ female mice developed normally and formed side branches before pregnancy. As seen in normal mice (23, 24), the Pin1$^{+/+}$ females underwent the normal massive expansion during pregnancy; the mammary epithelial ducts significantly extended their side branches and built up numerous alveolar structures, which replaced the mammary fat pad and formed lobules (Fig. 3 C and E). In sharp contrast to this normal pregnancy-induced response, in Pin1$^{-/-}$ female mice a severe reduction in mammary epithelial duct development was observed during pregnancy, and the mammary gland failed to undergo the usual massive expansion (Fig. 3 D and F). Consistent with these whole mount results, histological sections revealed that the pregnant wild-type mice displayed a massive proliferation of full-developed mammary epithelial cells (Fig. 3G). In contrast, the mammary gland of Pin1$^{-/-}$ pregnant mice showed a severe impairment in the development and proliferation of mammary epithelial cells (Fig. 3H).

Effects on Cyclin D1 Levels in Various Tissues. To detect the cyclin D1 protein in retina, we used immunostaining with affinity-purified anti-cyclin D1 antibodies. The cyclin D1 antibodies strongly stained the wild-type retina of 4–16 months of age (Fig. 4A; data not shown), which is consistent with high levels of cyclin D1 mRNA in
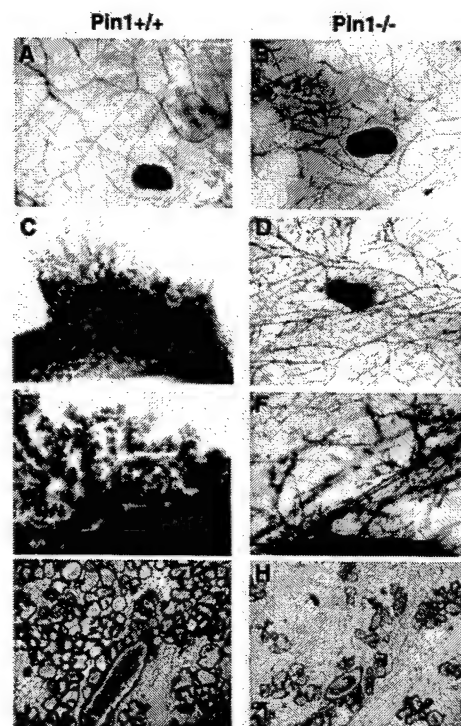
Fig. 3. Impaired mammary epithelial expansion during pregnancy in Pin1$^{-/-}$ mice. The whole mount (*A–F*) and histological (*G* and *H*) appearance of mammary glands derived from 3–4-month-old wild-type (*A, C, E,* and *G*) or Pin1$^{-/-}$ (*B, D, F,* and *H*) mice of ~4 months of age. The whole mounts of inguinal mammary glands were prepared, and the epithelial component was stained with carmine red. Histological sections were stained with hematoxylin and eosin. (*A*) Nulliparous wild-type mouse. (*B*) Nulliparous mutant mouse. (*C, E,* and *G*) Wild-type mouse, 1 day after delivery. (*D, F,* and *H*) Mutant mouse, 1 day after delivery.

this tissue as shown by *in situ* hybridization (23). In contrast, the antigenic peptide-blocked antibodies failed to detect any specific signal on retinal sections (Fig. 4A). In addition, the strong Pin1-staining signals obtained with affinity-purified anti-Pin1 antibodies (13) on wild-type tissues were in contrast to the lack of staining of Pin1$^{-/-}$ tissues (Fig. 4 B and C), confirming that Pin1$^{-/-}$ mice do not express Pin1 protein. Similar strong staining patterns were observed for both Pin1 and cyclin D1 in the retina (Fig. 4 B and C). More importantly, the cyclin D1 protein level in Pin1-deficient mice was strikingly lower than that in the age-matched wild-type mice both in the retina and mammary glands in pregnant mice (Fig. 4 B and C). To examine the effects on cyclin D1 levels in other tissues, several selected tissues from wild-type and Pin1$^{-/-}$ mice were subjected to immunoblotting analysis with anti-cyclin D1 antibodies. Although cyclin D1 levels appeared not to be affected in heart and kidney, cyclin D1 was significantly reduced in testis, spleen, liver, and lung in Pin1-deficient mice (Fig. 4D). These results indicate that mice lacking Pin1 display a significant reduction in cyclin D1 protein level in many tissues, including retina, spleen, and testis, and in the mammary gland in pregnant females, and suggest that Pin1 affects cyclin D1 levels in tissues that contain actively dividing cells.

Pin1 Regulates Cyclin D1 Turnover and Subcellular Localization in Addition to Its Transcription. The above results indicate that Pin1 loss-of-function in the mouse resembles many of the cyclin D1-null mouse phenotypes. Our previous studies have shown that Pin1

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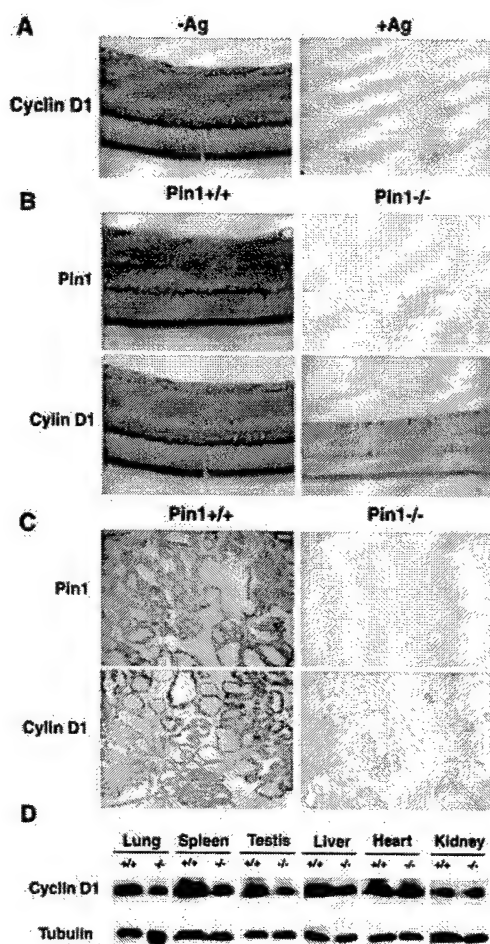
Fig. 4. Expression of Pin1 and cyclin D1 in various mouse tissues. (*A*) Specificity of immunostaining with anti-cyclin D1 antibodies. Anti-cyclin D1 antibodies that raised against the C-terminal peptide of cyclin D1 were affinity purified by using the cyclin D1 peptide. A retinal paraffin section was stained with the affinity-purified anti-cyclin D1 antibodies in the absence (*Left*) or presence (*Right*) of the cyclin D1 peptide (Ag) that was used as the antigen. (*B* and *C*) Sections of retina (*B*) and mammary gland in pregnant females (*C*) derived from Pin1$^{+/+}$ and Pin1$^{-/-}$ mice were stained with affinity-purified anti-Pin1 or anti-cyclin D1 antibodies. (*D*) Immunoblotting analysis of selected tissues with anti-cyclin D1 antibodies. Several selected tissues from Pin1$^{+/+}$ and Pin1$^{-/-}$ mice were lysed in SDS-sample buffer and subjected to immunoblotting analysis with anti-cyclin D1 antibodies. The same membranes were also probed with anti-tubulin antibodies as a loading control.

enhances the transcription of cyclin D1 through c-Jun/AP-1 and β-catenin/TCF in cancer cells. However, mouse models in which AP-1 or β-catenin/APC function is perturbed do not display strong cyclin D1-related phenotypes (30–32). This led us to speculate that an additional molecular mechanism may contribute to the drastic phenotypes observed in the Pin1-deficient mice.

To test this hypothesis, we first examined cyclin D1 mRNA and protein levels in Pin1$^{-/-}$ embryonic fibroblasts (MEFs). The cyclin D1 mRNA level was lower in Pin1$^{-/-}$ MEFs than that in wild-type MEFs (Fig. 5*A*), consistent with Pin1 being involved in regulation of cyclin D1 transcription (21, 22). However, the cyclin D1 protein level was decreased to a significantly greater extent in Pin1$^{-/-}$ MEFs (Figs. 5*B* and 6*A*), suggesting that Pin1 might also affect the

Fig. 5. Pin1 binds cyclin D1 phosphorylated on Thr-286 and stabilizes cyclin D1 protein. (*A*) Total RNA was isolated from wild-type (Pin1$^{+/+}$) or Pin1$^{-/-}$ 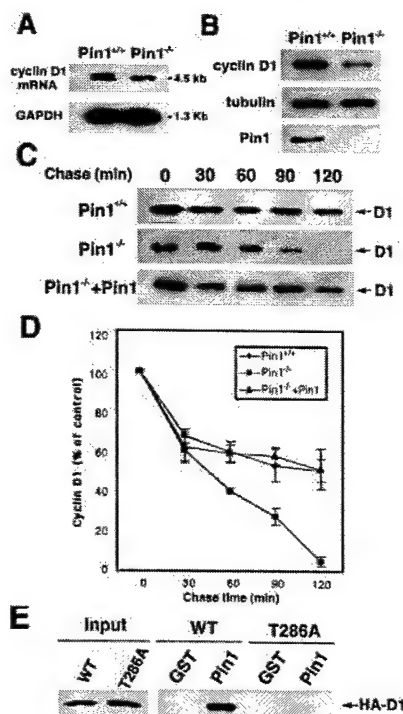MEFs and then subjected to Northern blot analysis, with glyceraldehyde-3-phosphate dehydrogenase as a loading control. (*B*) Reduced cyclin D1 protein in Pin1$^{-/-}$ MEFs. The same MEFs as described in *A* were subjected to immunoblot analysis with anti-cyclin D1, anti-tubulin, and anti-Pin1 antibodies. (*C* and *D*) Pin1 stabilizes cyclin D1 protein. Pin1$^{+/+}$, Pin1$^{-/-}$ MEFs, or Pin1$^{-/-}$ MEFs engineered to express Pin1 were transfected with HA-tagged cyclin D1 and CDK4. After 16 h, cells were metabolically labeled with [^{35}S]Met for 40 min. Cells were washed with complete medium containing excess unlabeled Met and collected at indicated times. Cells were lysed and immunoprecipitated with 12CA5 antibody, followed by autoradiography (*C*). The radioactivity of immunoprecipitated cyclin D1 was quantified with a Phosphoimager and normalized to the 0-h point. Results shown are means ± SD for three independent experiments (*D*). (*E*) Pin1 binds cyclin D1 phosphorylated on Thr-286. Cells were transfected with either wild-type HA-tagged cyclin D1 or HA-tagged cyclin D1 mutant (T286A) for 24 h. Cell extracts were incubated with glutathione agarose beads containing GST or GST-Pin1. After washing, binding proteins were subjected to immunoblotting analysis with 12CA5 mouse monoclonal antibody to HA peptide.

stability of cyclin D1 protein. To test this idea, we investigated the stability of exogenously expressed cyclin D1 by using pulse–chase experiments. Although the stability of cyclin D1 in wild-type MEFs was almost the same as those in other cells reported earlier (26, 27), its stability in Pin1$^{-/-}$ MEFs was significantly decreased (Fig. 5 *C* and *D*). Moreover, reexpression of Pin1 in Pin1$^{-/-}$ MEFs restored the stability of cyclin D1 (Fig. 5 *C* and *D*). These results indicate that Pin1 increases the stability of cyclin D1 protein.

Because previous evidence indicates that the phosphorylation on Thr-286 by GSK-3β is a critical factor for cyclin D1 localization and turnover (26, 27), we asked whether Pin1 binds cyclin D1 phosphorylated on Thr-286 and whether Pin1 affects its subcellular localization. GST-pull down analyses revealed that Pin1 directly bound to cyclin D1 but not to its T286A mutant (Fig. 5*E*), indicating that Pin1 binds to cyclin D1 likely via phosphorylated Thr-286, which is succeeded by a proline and resided in a consensus Pin1-binding sequence (7, 9). Furthermore, in exponentially grow-
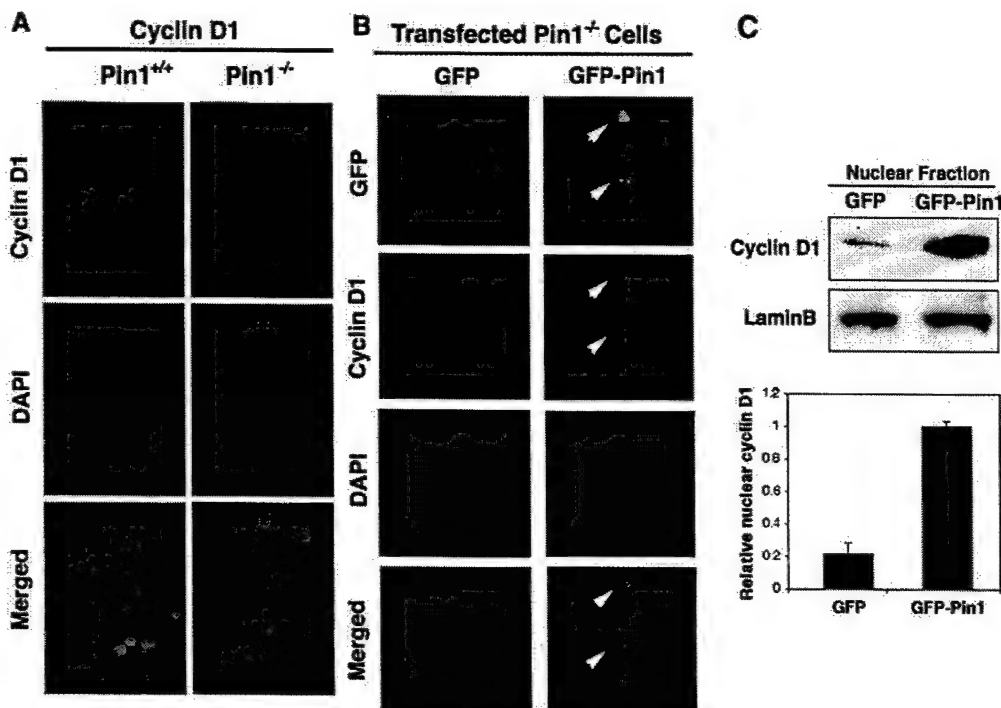
Fig. 6. Pin1 increases cyclin D1 protein level and increases its nuclear localization. (*A*) Reduced cyclin D1 in Pin1$^{-/-}$ MEFs. Growing wild-type or Pin1$^{-/-}$ MEFs were fixed and stained with anti-cyclin D1 antibodies and DAPI. (*B*) Pin1 increases the nuclear localization of cyclin D1. Pin1$^{-/-}$ MEF cells transfected with either GFP or GFP-Pin1 were arrested in G$_0$ by serum deprivation and contact inhibition, and then cells were harvested at 18 h after serum addition. Subcellular localization of cyclin D1 was determined by immunostaining with cyclin D1 antibodies and DAPI. Arrows point to GFP-Pin1-expressing cells. (*C*) The nuclear fraction was isolated from cells transfected as described in *B* by using hypotonic buffer and then subjected to immunoblotting analysis with anti-cyclin D1 and anti-lamin B antibodies. Relative amounts of nuclear cyclin D1 was semiquantified and normalized with lamin.

ing Pin1$^{-/-}$ MEFs, the level of endogenous cyclin D1 was not only significantly reduced compared with that in wild-type MEFs, but cyclin D1 was also primarily localized in cytoplasm of Pin1$^{-/-}$ MEFs, whereas it was largely nuclear in wild-type MEFs (Fig. 6*A*). In addition, the cyclin D1^{T286A} mutant remained in the nucleus even in Pin1$^{-/-}$ MEFs (data not shown) (28). These results suggest that Pin1 might affect localization of cyclin D1. To confirm this, we looked at the cyclin D1 localization during S phase, when cyclin D1 is known to be exported to cytoplasm (26–28). Pin1$^{-/-}$ MEFs transfected with either GFP or GFP-Pin1 were arrested in G$_0$ by serum starvation and allowed to enter the cell cycle by the addition of serum. At 18 h after the serum addition, when cells were in S phase, as monitored by BrdU incorporation (data not shown), cells were fixed and subjected to immunostaining with cyclin D1 antibodies (Fig. 6 *A* and *B*), or the nuclear fraction was isolated and subjected to immunoblotting analysis with cyclin D1 antibodies (Fig. 6*C*). As shown (26, 27), cyclin D1 was primarily localized in the cytoplasm in nontransfected or GFP-transfected Pin1$^{-/-}$ MEFs (Fig. 6 *A* and *B*). In contrast, cyclin D1 was mainly localized in the nucleus in over 90% GFP-Pin1-transfected cells (Fig. 6*B*, data not shown). Furthermore, levels of cyclin D1 in the nucleus were significantly higher in GFP-Pin1-transfected cells than control GFP-transfected Pin1$^{-/-}$ MEFs (Fig. 6*C*). Together, these results indicate that Pin1 regulates the turnover and subcellular localization of cyclin D1.

Discussion

We report here that Pin1$^{-/-}$ mice display many severe phenotypes, including decreased body weight, retinal degeneration,

mammary gland retardation, and testicular atrophy. Most of these phenotypes are remarkably similar to those of cyclin D1-deficient mouse phenotypes. Of several phenotypes observed in Pin1$^{-/-}$ mice, the alterations in retina and mammary gland seemed to be most drastic. We found that Pin1$^{-/-}$ mice show dramatic impairments in cell survival or proliferation in the retina, especially at old age. Moreover, it is very clear and striking that in pregnant Pin1$^{-/-}$ female, mammary epithelia cells fail to undergo massive proliferation in the development of alveolar structures and ductal side branching. Our study demonstrated that Pin1 is highly expressed in retina and mammary gland compared with other tissues (data not shown), and the depletion of Pin1 causes a dramatic retinal atrophy and mammary gland impairment. Furthermore, disruption of Pin1 affects the level of cyclin D1 in the tissues that contain actively proliferative cells, such as spleen, retina, and mammary gland in pregnant females. Moreover, cyclin D1$^{-/-}$ mice also display a very similar phenotype in the retina and mammary gland. These results suggest that Pin1 could play an essential role in maintaining survival or proliferation of cells through regulating cyclin D1 expression. One notable difference between Pin1- and cyclin D1-deficient mice is that cyclin D1 mutant mice display a dramatic reduction in retinal layers at an early stage development because of proliferative failure (23, 24). However, in Pin1$^{-/-}$ mice, the thickness of the retina decreases slowly with age and becomes pronounced by over 1 year of age. These results indicate that the retinal hypoplasia in Pin1$^{-/-}$ mice is not because of proliferative failure during the embryonic development but rather likely because of a failure of cells to maintain cell

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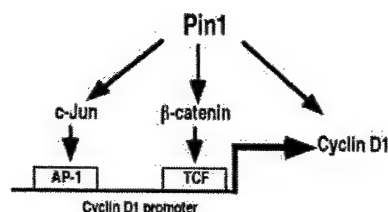
Fig. 7. A model for regulation of cyclin D1 function by Pin1. In addition to that Pin1 enhances cyclin D1 promoter activity through AP-1 and TCF sites as a result of activation and/or stabilization of phosphorylated c-Jun and β-catenin, respectively, our current results demonstrate that Pin1 directly regulates the stability and subcellular localization of cyclin D1 itself.

survival and/or proliferation after birth because there is no obvious difference in the retina between Pin1$^{-/-}$ and wild-type mice at birth. This might be due to the fact that cyclin D1 level in Pin1$^{-/-}$ mice is lower, but not completely absent as in the case of cyclin D1 knockout mice. Thus, this level of cyclin D1 may become a limiting factor with aging and thereby affect survival or cell proliferation.

Our results strongly suggest that Pin1 regulates cyclin D1 not only through transcriptional regulation but also via a posttranslational mechanism. Previous studies by using human cancer tissues and cancer cell lines have demonstrated that Pin1 enhances cyclin D1 promoter activity through AP-1 and TCF sites as a result of activation and/or stabilization of phosphorylated c-Jun and β-catenin, respectively (21, 22). Here, we demonstrate that Pin1 also regulates the stability and subcellular localization of cyclin D1 itself. Phosphorylation of cyclin D1 on the Thr-286 site regulates turnover and localization of cyclin D1 by enhancing its nuclear export, which leads to degradation of cyclin D1 in the cytoplasm (26, 27). We have shown that Pin1 can bind to the phosphorylated Thr-286–Pro motif in cyclin D1 and stabilize cyclin D1, presumably by preventing nuclear export of cyclin D1 and proteolysis in cytoplasm. Interestingly, Pin1 regulates the turnover and localization of β-catenin in a similar manner (22). Thus, Pin1 positively regulates cyclin D1 function at both transcriptional and posttranslational levels (Fig. 7), and this may explain why Pin1 loss-of-function in the mouse

resembles the cyclin D1-null phenotypes. Because cyclin D1 plays a critical role in oncogenesis (23, 24, 33–38), our current results further support a role of Pin1 in breast cancer (21, 22).

Pin1 has been demonstrated to have many functions; however, we have demonstrated here that Pin1$^{-/-}$ mice display rather restricted phenotypes that are related to those observed in cyclin D1$^{-/-}$ mice. The lack of more severe phenotypes can be explained by the idea that there are other Pin1-like genes that can compensate for the functions of Pin1. This idea is supported by our recent isolation of a second Pin1-like gene from *Drosophila* (A.R. and K.P.L., unpublished data). This finding strongly suggests the possibility of other existing Pin1-like genes in the higher species such as mouse and human. The strikingly testicular atrophy, which is not found in cyclin D1-null mice but seen in cyclin D2 knockout mice (39) or the cyclin D-dependent kinase inhibitors (40, 41), suggests that Pin1 may target other proteins, perhaps other D-type cyclins. Further studies are needed to illustrate the role of Pin1 in spermatogenesis.

In summary, we report that mice lacking Pin1 do display a range of severe cell-proliferative abnormalities, many of which resemble those in cyclin D1-deficient mice. Furthermore, disruption of Pin1 also causes a striking reduction in cyclin D1 level in many tissues, including the retina and mammary epithelial cells of pregnant females, the two most affected tissues. Using Pin1$^{-/-}$ MEF cells, we demonstrate that Pin1 binds and stabilizes cyclin D1 and increases its nuclear localization in addition to affecting cyclin D1 transcription. These results provide insight for an essential role of Pin1 in maintaining cell proliferation and regulating cyclin D1.

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Role of Pin1 in the Regulation of p53 Stability and p21 Transactivation, and Cell Cycle Checkpoints in Response to DNA Damage

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Running Title: Role of Pin1 in DNA Damage Response

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ABSTRACT

DNA damage leads to stabilization and accumulation of p53, which plays a pivotal role in transcriptional activation of p21 and cell cycle arrest. The increase in p53 stability depends critically on its phosphorylation on serine/threonine residues, including those preceding a proline (pSer/Thr-Pro). The pSer/Thr-Pro moiety exists in the two distinct *cis* and *trans* conformations and their conversion is catalyzed specifically by the prolyl isomerase Pin1. Pin1 regulates the conformation and function of certain phosphorylated proteins and plays an important role in cell cycle regulation, oncogenesis and Alzheimer's disease. However, nothing is known about the role of Pin1 in DNA damage. Here we found that DNA damage enhanced the interaction between Pin1 and p53, which depended on the WW domain in Pin1 and $Ser^{33/46}$-Pro motifs in p53. Furthermore, Pin1 regulates the stability of p53 and its transcriptional activity towards the p21 promoter. As a result, p53 and p21 barely increased after DNA damage in Pin1 knockout embryonic fibroblasts or in neoplastic cells depleted of Pin1. Moreover, Pin1 null cells displayed significant defects in cell cycle checkpoints induced by DNA damage. These results demonstrate a new role of Pin1 in regulating p53 function during DNA damage.

INTRODUCTION

The tumor suppressor protein p53 regulates multiple cellular functions, including cell cycle checkpoint, genomic stability, transcriptional activation of specific subsets of genes and apoptosis (1-6). DNA damage induced by such as ionizing radiation leads to stabilization and accumulation of p53 (1-6). This p53 induction plays a pivotal role in transcriptional activation of the cell cycle inhibitor p21 and cell cycle arrest (7-9) and is at least partially due to its dissociation from the ubiquitin ligase MDM2 (10,11). The increases in the p53 stability and/or transcriptional activity depend critically on its phosphorylation on multiple serine/threonine residues, including those preceding a proline (pSer/Thr-Pro) (12-19). Therefore, Pro-directed phosphorylation is a critical mechanism for activation of p53 upon DNA damage. However, it is not clear whether the stability and/or function of p53 are regulated after Pro-directed phosphorylation.

Interestingly, pSer/Thr-Pro motifs in proteins exist in *cis* and *trans* conformations, whose conversion is normally inhibited by phosphorylation, but is specifically catalyzed by the prolyl isomerase Pin1 (20-23). Pin1 contains an N-terminal WW domain and C-terminal isomerase domain. The WW domain binds specific pSer/Thr-Pro motifs and targets Pin1 to a subset of phosphoproteins where the PPIase domain induces conformational changes by isomerizing specific pSer/Thr-Pro bonds. Such conformational changes have profound effects on catalytic activity, dephosphorylation, protein-protein interactions, subcellular location, and/or stability of Pin1 substrates (21,22,24-34). Functionally, Pin1-mediated mechanism has been shown to regulate several phases of the cell cycle, including G1/S and G2/M as well as the DNA replication checkpoint (35). Furthermore, Pin1 is strikingly overexpressed in many human cancers and affects the stability of certain phosphoproteins, such as cyclin D1 and β-catenin (32-34). Therefore, phosphorylation-specific prolyl isomerization is a new mechanism for regulating function of certain phosphorylated proteins. However, it is not known whether this mechanism is important in DNA damage response. Here we report that Pin1 binds phosphorylated p53, and is important for p53 stabilization, p21 transactivation and cell cycle checkpoints in response to DNA damage.

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MATERIALS AND METHODS

Cell Culture, DNA constructs and Transfection --- Primary embryonic fibroblasts (MEFs) were prepared from 15-16 day-old embryos of WT and Pin1^{-/-} mice, as described (34). They were transfected using Effectene (Quiagen). Breast cancer cells MCF7 and T43D cells were transfected using Fugene 6 (Boehringer), as described (32). Constructs expressing Pin1, its point mutants or Pin1[AS] have been described (21,23). p53, its dominant-negative mutant (R248W), as well as the phoshorylation site mutants Ser33 and Ser46 expression constructs and the p21 promoter luciferase constructs were as described (7, 16).

GST Pulldown, Immunoprecipitation and Immunoblotting Analyses --- To examine the interaction between Pin1 and p53, cells were irradiated with 5 Gy of γ-ray or mock-irradiated, as described *(36,37)*. The cells were harvested 4 hr later and lysed in a lysis buffer containing 1% Triton X100. The supernatants were subjected to GST-Pin1 pulldown assay, followed by immunoblotting analysis using monoclonal antibodies against p53 (Ab-6 for human p53 or Ab-3 for murine p53)(Oncogene), as described (25,27,32). For co-immunoprecipitation, we used agarose-conjugated anti-p53 polyclonal antibodies (SantaCruz), with unrelated antibodies (Pericentrin antibodies) as a control, as described (25,27,32).

Promoter Reporter Assays --- Promoter reporter assays were performed with the Dual-luciferase reporter assay system (Promega) at 24 hrs after transfection, as described (32). Since Pin1[AS] induces mitotic arrest and apoptosis at 48-72 hr after transfection (21), all experiments with Pin1[AS] were performed before 36 hr after transfection, when no significant apoptotic cells were observed, as described (21,32).

Cell Cycle Analysis --- To determine the cell cycle profile after DNA damage, cells were harvested at 24 hr after irradiation, followed by propidium iodide staining and flow cytometric analysis in a FACScan using the CellQuest Software Package (Becton Dickinson), as described (38,39).

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RESULTS

DNA Damage Enhances the Interaction between Pin1 and p53

Pin1 has been shown to bind and regulate the function of a certain subset of phosphorylated proteins (20,33,34,40). Interestingly, DNA damage induces phosphorylation of p53 on several Ser/Thr sites, including Ser33-Pro and Ser46-Pro motifs and these phosphorylation sites are important for p53 function (1-6,12-19). Moreover, Ser33 and Ser46 have multiple upstream hydrophobic residues (Leu, Val, and/or Met) and are preceded by a Pro residue, the two most critical features for Pin1 preferred binding sites (22). Therefore, it is conceivable that Pin1 might bind p53 and regulate its function following DNA damage.

To test this possibility, we first asked whether Pin1 binds p53 using GST-Pin1 pulldown analysis (22,25-27). As shown previously (41), when T47D human breast cancer cells, which contain wild-type p53 protein, were treated with 5 Gy of γ-radiation, p53 levels increased significantly within 6 hrs (Fig. 1A). More importantly, GST-Pin1, but not GST alone, pulled down p53 from mock-irradiated cells and the binding was significantly increased after cells were irradiated (Fig. 1A), even after normalizing p53 loading (Fig. 1B). These results indicate that exogenous Pin1 can bind cellular p53, which is enhanced by DNA damage. To confirm this result, we performed coimmunoprecipitation experiments to detect the endogenous complex. Anti-p53 antibodies, but not control antibodies, immunoprecipitated endogenous Pin1 and this co-immunoprecipitation was also significantly increased by DNA damage (Fig. 1C, D). These results indicate that DNA damage induces the interaction between Pin1 and p53.

Next we tested whether the binding of Pin1 to p53 depends on the WW domain of Pin1 and the phosphorylation of p53 on specific Ser-Pro motifs, as shown for other Pin1 substrates (20,22,25,27). As indicated above, primary sequence analysis suggests that two potential Pin1 binding sites in p53 may be Ser33-Pro and Ser46-Pro motifs. We therefore used p53 single point mutants Ser33Ala or Ser46Ala as well as a double mutant Ser33/46Ala in Pin1 binding assays. These experiments were performed in HeLa cells because these cells very little endogenous p53 protein (Fig. 1E), due to high levels of viral oncoprotein E6, and therefore have successfully been

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used to analyze the specific effects of overexpression of p53 and its mutants previously (42,43). To increase phosphorylation on Ser33 and Ser46, cell lysates were obtained 4 hrs after ionizing radiation with 5 Gy, at a time when Ser33 is shown to be significantly phosphorylated (44). Consistent with the absence of functional p53 in these cells, no detectable p53 was pulled-down by Pin1 from vector-transfected control cells. However, a substantial amount of transfected wildtype p53 pulled down by Pin1, but not by its mutant containing the Ser16Glu mutation (Fig. 1F). Given that the Ser16Glu mutation disrupts the ability of the Pin1 WW domain to bind phosphoproteins, these results confirm that Pin1 binds p53 via its WW domain. Furthermore, Pin1 binding to the Ser33Ala or Ser46Ala p53 single point mutant was considerably decreased, while no binding was detected between Pin1 and the Ser33/46Ala double mutant of p53 (Fig. 1F), indicating the requirement of both Ser residues for the binding. The above results indicate that DNA damage enhances the specific interaction between Pin1 and p53 and the binding depends on the WW domain in Pin1 as well as the Ser33 and Ser46 residues in p53.

Effects of Pin1 on the protein stability of p53 upon DNA damage

We next asked whether Pin1 affects p53 accumulation and p53-dependent responses after DNA damage. To address the first question, we used primary embryonic fibroblasts (MEFs) derived from wild type (WT) or Pin1 knockout (Pin1$^{-/-}$) mice. When WT MEFs were irradiated, p53 levels strongly increased while Pin1 levels remained basically unchanged (Fig. 2A and C, first panel). This DNA damage-induced p53 accumulation was time- as well as dose-dependent, (Fig. 2A and B), as shown previously (45-47). However, this DNA damage-induced p53 accumulation was markedly suppressed in Pin1$^{-/-}$ MEFs (Fig. 2A and B). Furthermore, both the usual time- and dose-dependent increase in p53 levels in response to ionizing radiation was abolished in Pin1$^{-/-}$ MEFs (Fig. 2A and B). These results indicate that p53 protein fails to increase in Pin1$^{-/-}$ MEFs, in contrast to that in WT MEFs. To further support that Pin1 is important for p53 accumulation following DNA damage, we depleted Pin1 from WT MEFs via expression of Pin1 antisense construct (Pin1^{AS}), which has been shown to effectively deplete Pin1 in many different cell types

(21,32,34). Indeed, depletion of Pin1 from WT MEFs drastically suppressed induction of p53 after DNA damage (Fig. 2C). These results indicate that Pin1 is required for the accumulation of p53 after DNA damage.

The above results suggest that Pin1 may be required for the stabilization of p53 after DNA damage. To examine this possibility, we examined the effects of Pin1 on p53 stability after DNA damage using a cycloheximide chase, as described (44). Pin1$^{-/-}$ MEFs were cotransfected with p53 and control vector, Pin1 or the S16E Pin1 mutant that failed to bind p53 (Fig. 1F). After irradiation, cycloheximide was added to inhibit de novo p53 synthesis and the steady-state levels of p53 were determined using antibodies that detected only transfected human p53 (Fig. 2D, E). In Pin1$^{-/-}$ MEFs transfected with the control vector, the half-life of p53 was about 60 min (Fig. 2D, E). However, steady-state levels of p53 were higher before the addition of cycloheximide in Pin1$^{-/-}$ MEFs transfected with Pin1 than vector controls (Fig. 2D). Importantly, the half-life of p53 was significantly increased to ~ 6 hr by expression of Pin1. In contrast, the Pin1 mutant that failed to bind p53 did not have detectable effect on the p53 stability. These results demonstrate that the binding of Pin1 to p53 is essential for Pin1 to increase p53 stability after DNA damage.

To further support that phosphorylation-dependent association is important for Pin1 to stabilize p53, we examined the stability of the mutant S33/46A p53 mutant that failed to bind Pin1 (Fig. 1F). As shown in the newly added Fig. 2E and 2F, In Pin1$^{-/-}$ MEFs, Pin1 stabilized p53, but not its mutant S33/46A mutant after DNA damage (Fig. 1F). The above results indicate that Pin1 and its phosphorylation-dependent association is required for stabilization of p53 and also reveal an important new role of phosphorylation of Ser33 and Ser46 in regulating the stability of p53.

Effects of Pin1 on p53 transactivation towards p21 after DNA Damage

To further investigate the consequences of the Pin1 and p53 interaction, we next assayed the effects of Pin1 on p53-dependent responses. Since one of the major downstream targets of p53 is p21, we first examined whether Pin1 affects p53-dependent p21 transactivation without DNA damage given the detectable, although weak, interaction (Fig. 1A-D). As shown in Fig. 3A, Pin1 alone had a

modest stimulatory effect on the p21 promoter activity. This stimulatory effect was significantly enhanced when co-transfected with p53, but was inhibited by dominant-negative p53 (Fig. 3A). In contrast, depletion of Pin1 by Pin1[AS] blocked the ability of p53 to activate the p21 promoter (Fig. 3A). Moreover, a mutation in the p53-binding site in the p21 promoter completely abolished the effects of Pin1 on the p21 promoter (Fig. 3B). These results show that Pin1 can enhance the ability of p53 to activate the p21 promoter.

Since the Pin1 and p53 interaction is enhanced after DNA damage, we next examined the effects of irradiation on the ability of Pin1 to enhance the p21 promoter. As shown in Fig. 3B, irradiation increased p21 transactivation about 2-4 times after transfection with Pin1 alone in comparison to vector control. However, p21 transactivation in response to irradiation was greatly enhanced when Pin1 and p53 were cotransfected (Fig. 3B). This stimulatory effect of Pin1 and p53 depended on DNA damage since p21 transactivation is 3-4 times stronger in the Pin1 or Pin1 and p53 cotransfected radiated cells in comparison to the mock-radiated control ((Fig. 3B). Again, the mutation in the p53-binding site in the p21 promoter completely abolished the effects of Pin1 on the p21 promoter (Fig. 3B). Similarly, a dominant-negative p53 mutant significantly suppressed the Pin1's effects (Fig. 3B). These results indicate the critical role of p53 in mediating the Pin1's effect on the p21 promoter. Finally, the Pin1 point mutant that either could not bind p53 (Pin1[S16E], Pin1[W34A]) or isomerize pSer/Thr-Pro motifs (Pin1[K63A]) failed to increase p53 transactivation (Fig. 3B). These results indicate that Pin1 enhances the p53-dependent p21 transactivation upon DNA damage and that both Pin1's WW domain and its PPIase activity are required for this cooperation.

If Pin1 enhances the p53-dependent p21 transactivation upon DNA damage, it would be expected that inhibition of Pin1 might affect the accumulation of p21 after DNA damage. Therefore, we compared the DNA damage-induced p21 accumulation in the presence or absence of Pin1. As shown previously (45), p21 levels are strongly increased in WT MEFs after ionizing radiation and this induction was time-dependent as well as dose-dependent (Fig. 2A-C). However, under the same conditions, p21 levels failed to increase in Pin1^{-/-} MEFs or WT MEFs that were depleted of Pin1 by Pin1[AS] (Fig. 2A-C). Furthermore, both the time- and dose-dependent increase in p21 levels

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was abolished in Pin1$^{-/-}$ MEFs (Fig.2A-B). These results demonstrate that loss of Pin1 abolishes the accumulation of p53 and p21 after DNA damage. Of note, Pin1 appeared to have more profound effects on p21 protein levels than on its promoter and that p21 stability is also regulated by Pro-directed phosphorylation (48), Pin1 might also directly regulate p21 stability.

Effects of Pin1 on DNA Damage-induced Cell Cycle Checkpoints

Given that Pin1 is required for accumulation of p53 and p21 after DNA damage, an important question is whether Pin1 affects cell cycle checkpoint control in response to DNA damage. To address this question, we subjected WT or Pin1$^{-/-}$ MEFs to 5 Gy of gamma irradiation. The cell cycle phenotypes were analyzed by flow cytometry in addition to direct microscopic examination. Before DNA damage, there were not obvious differences between WT and Pin1$^{-/-}$ MEFs with the exception that Pin1$^{-/-}$ cells had slightly more cells in G1 and fewer cells in S phase, as compared with WT cells (Fig. 4A, B). This phenotype is consistent with the previous findings that Pin1 is an important regulator of the G1/S activator cyclin D1 (34).

Importantly, obvious differences in cell morphologies and cell cycle profiles were observed after DNA damage. As compared with those before irradiation, WT MEFs displayed interphase cell morphologies, whereas significantly more Pin1$^{-/-}$ MEFs were rounded up (data not shown). These differences were further confirmed by flow cytometrical analysis (Fig. 4). 12 hr after irradiation, WT MEFs in S phase were dramatically reduced and the major of cells were accumulated with the 4n DNA content (Fig. 4), confirming intact cell cycle checkpoints in WT MEFs, as shown (45-47). However, Pin1$^{-/-}$ MEFs in S phase were not changed. Furthermore, DNA-damage-induced G2 arrest was significantly inhibited in Pin1$^{-/-}$ MEFs (Fig. 4). These results indicate that loss of Pin1 leads to multiple cell cycle checkpoint defects in response to DNA damage.

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DISCUSSION

Here we report that Pin1 plays an important role in regulating p53 function and in modulating cellular response to DNA damage. Pin1 bound p53 and also increased its protein stability, which depended on the binding of Pin1 to phosphorylated Ser33/46 of p53. Furthermore, Pin1 potentiated the transcriptional activity of p53 towards the p21 promoter, which required the function of both the WW domain and the PPIase domain of Pin1 as well as the presence of the p53-binding site in the p21 promoter. Moreover, p53 and p21 barely increased after ionizing radiation in Pin1^{-/-} MEFs or in neoplastic cells depleted of Pin1. Finally, Pin1^{-/-} MEFs displayed significant defects in cell cycle checkpoints induced by DNA damage. Given that the well established role of p53 in the cell cycle checkpoint regulation in response to DNA damage, these results demonstrate that Pin1 is a novel regulator of p53 in DNA damage response.

It remains to be determined how Pin1 regulates the ability of p53 and p21 to upregulate during DNA damage. One of the major cellular responses to DNA damage is phosphorylation of p53, which leads to the activation of p53 by destabilizing its interaction with MDM2 and by increasing its transcriptional activity (1-6). Pin1 is a phosphorylation-specific PPIase that regulates the function of certain phosphorylated proteins by binding and isomerizing specific phosphorylated Ser/Thr-Pro motifs, whose isomerization is normally inhibited upon phosphorylation (20-22,24-34). We have shown that the Pin1-binding or isomerase mutant fails to increase the stability and transactivation activity of p53. Furthermore, the S33/46A mutant that fails to bind Pin1 fails to be stabilized after DNA damage. These results strongly suggest for the first time that phosphorylation of Ser33 or Ser46 plays an important role in regulating the stabilization of p53. This is consistent with the fact that both residues lie in immediate vicinity of the Mdm2 binding site and affect transcriptional activity of p53 (49-51). Therefore, we propose that when p53 is phosphorylated following DNA damage, Pin1 binds and isomerizes p53 on phosphorylated Ser33/46. This conformational change may prevent binding of p53 to its ubiquitin ligase MDM2, affect phosphorylation of p53 on other sites, and/or affect transcriptional activity of p53 toward p21.

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In summary, we demonstrate that following DNA damage Pin1 not only interacts with p53, but also increases its protein stability and transcriptional activity towards the p21 promoter. The biological significance of the Pin1 and p53 interaction is further substantiated by the findings that p53 and p21 barely increase in Pin1-deficient or depleted fibroblasts as well as that Pin1-deficient fibroblasts fail to undergo cell cycle arrest after DNA damage. These results indicate that Pin1 is a new regulator of p53 in DNA damage response.

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Figure Legends

Fig. 1. DNA Damage Enhances the Specific Interaction between Pin1 and p53.

(A, B) Interaction between Pin1 and p53. T47D cells were treated with 5 Gy γ-ray (+IR) or mock-treated (-IR) and cells were harvested 6 hr later. The same amount of lysates (A) or lysates normalized for p53 (B) were subjected to immunoblotting with anti-p53 antibodies directly (left), or after GST pulldown experiment with agarose beads containing GST or GST-Pin1 (right).

(C, D) Coimmunoprecipitation of Pin1 and p53. The same amount of lysates (C) or lysates normalized for p53 (D) as described in A and B were immunoprecipitated with anti-p53 or non-related antibodies (Control), followed by immunoblotting with anti-p53 or -Pin1 antibodies.

(E, F) Requirement of the WW domain in Pin1 as well as the Ser33 and Ser46 residues in p53 for their interaction. 24 hrs after transfection with p53 or its mutants, HeLa cells were irradiated, followed by immunoblotting analysis with anti-p53 antibodies (E). The same cell lysates were incubated with beads containing either GST, GST-Pin1 or GST-Pin1^{S16E} (E) and bound proteins were subjected to immunoblotting with p53 antibodies (F).

Fig. 2. Loss or Depletion of Pin1 Decreases p53 Stability and p21 Induction after DNA Damage.

(A, B) Pin1$^{-/-}$ MEFs lack the time- and dose-dependent increase of p53 and p21 after DNA damage. WT or Pin1$^{-/-}$ MEFs were irradiated with the indicated doses of γ-ray and then harvested 6 hr after irradiation (A), or with γ-ray and harvested at the indicated time points (B). Lysates were subjected to immunoblotting with antibodies against p53, p21 or Pin1, with actin or tubulin as a control.

(C) Lack of p53 and p21 induction after DNA damage in Pin1-inhibited MEFs. WT MEFs (first two panels) were transfected with either control vector or anti-sense Pin1 (Pin1^{AS}) for 24 hrs. Six hours after mock (-) or irradiation (+), cells were subjected to immunoblotting analysis.

(D, E) Requirement of Pin1 for stabilization of p53. Pin1$^{-/-}$ MEFs were co-transfected with p53 and control vector, Pin1 or its S16E mutant for 24 hours, followed by irradiation. 4 hrs after irradiation, cycloheximide was added and lysates were prepared at the indicated time points, followed by

immunoblotting with anti-human p53 (upper panel) or anti-actin (lower panel) antibodies (D). p53 levels were semi-quantified using Imagequant and normalized using actin as an internal control. For comparing the p53 stability in the absence or presence of Pin1 or its mutant, p53 levels at 0 was defined as 100%.

(F, G) Requirement of Ser33/46 for stabilization of p53. p53 or its mutant was cotransfected into Pin1$^{-/-}$ MEFs and their stability was assayed as described in D and E.

Fig. 3. Pin1 increases p53 transactivation towards the p21 promoter, which is enhanced by DNA damage.

(A) Cooperation between Pin1 and p53 in activating the p21 promoter. Cells were co-transfected with expression constructs for Pin1, Pin1^{AS}, WW domain Pin1 mutant (Pin1^{S16E}, Pin1 ^{W34A}) or PPIase domain Pin1 mutant (Pin1^{K63A}) and p53 or dominant-negative p53 (dnp53), together with a p21 luciferase reporter construct. The activity of the reporter luciferase was expressed in relative to the activity in control vector transfected cells, which is defined as 1.0.

(B) DNA damage enhances cooperation between Pin1 and p53 in activating the p21 promoter. Cells were co-transfected with indicated expression constructs and a p21 luciferase reporter construct. For the assays in the last two sets of experiments labeled as Mt p21 promoter, the same p21 luciferase construct but containing a mutated p53 binding site was used. 24 hr after transfection cells were mock-treated (white bars) or irradiated (solid bars), followed by luciferase assay.

Fig. 4. Loss of Pin1 Causes Prominent Defects in the Cell Cycle Checkpoints in Response to DNA Damage.

WT or Pin1$^{-/-}$ MEFs were subjected to mock or 5 Gy of irradiation. 12 hr later, the cells were harvested and stained with propidium iodide, followed by flow cytometry to determine the cell cycle profile (A) with the cell cycle distribution being presented in (B).
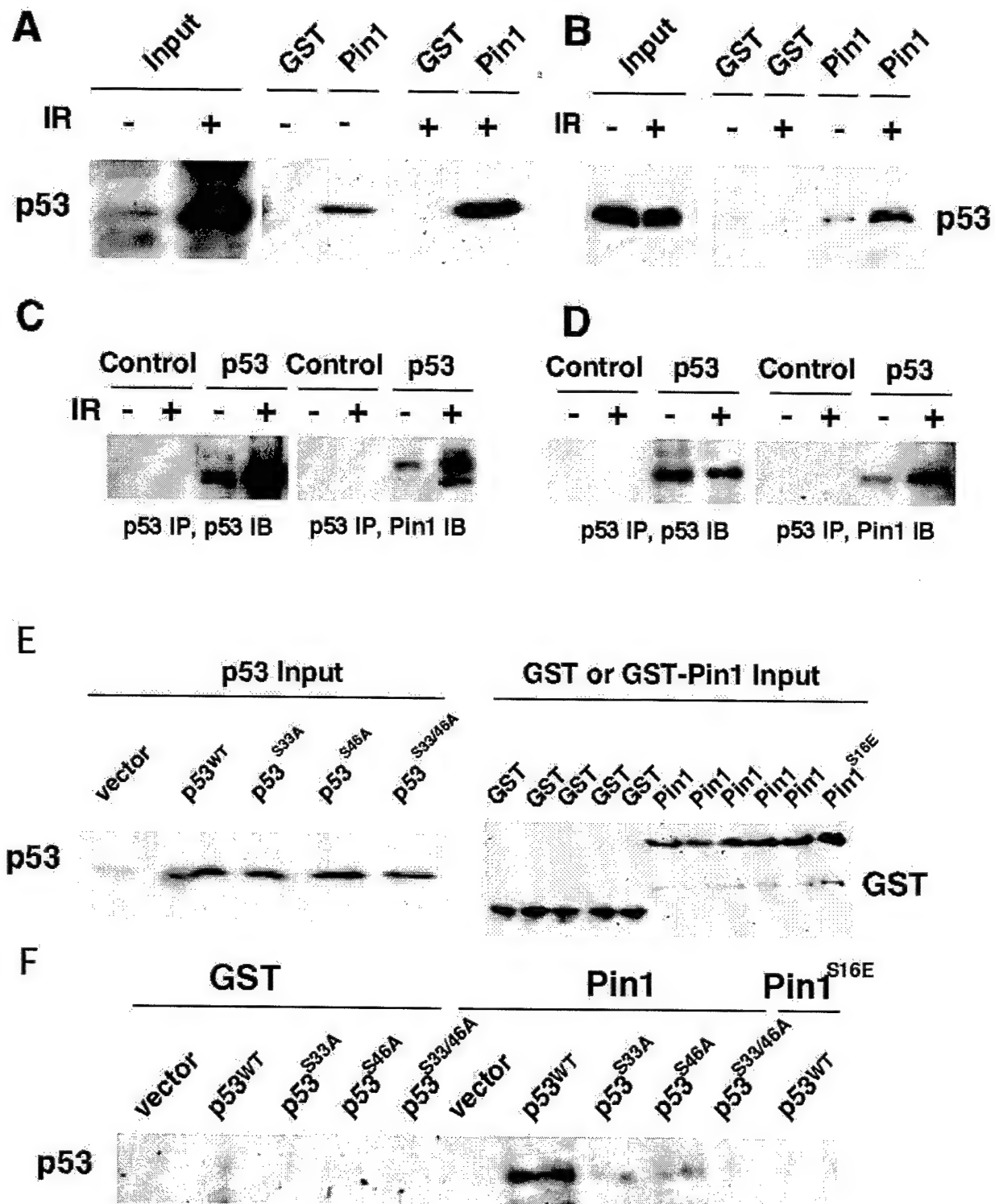
Wulf et al. Fig. 1

Wulf et al., Fig. 2

Wulf et al., Fig. 3

A
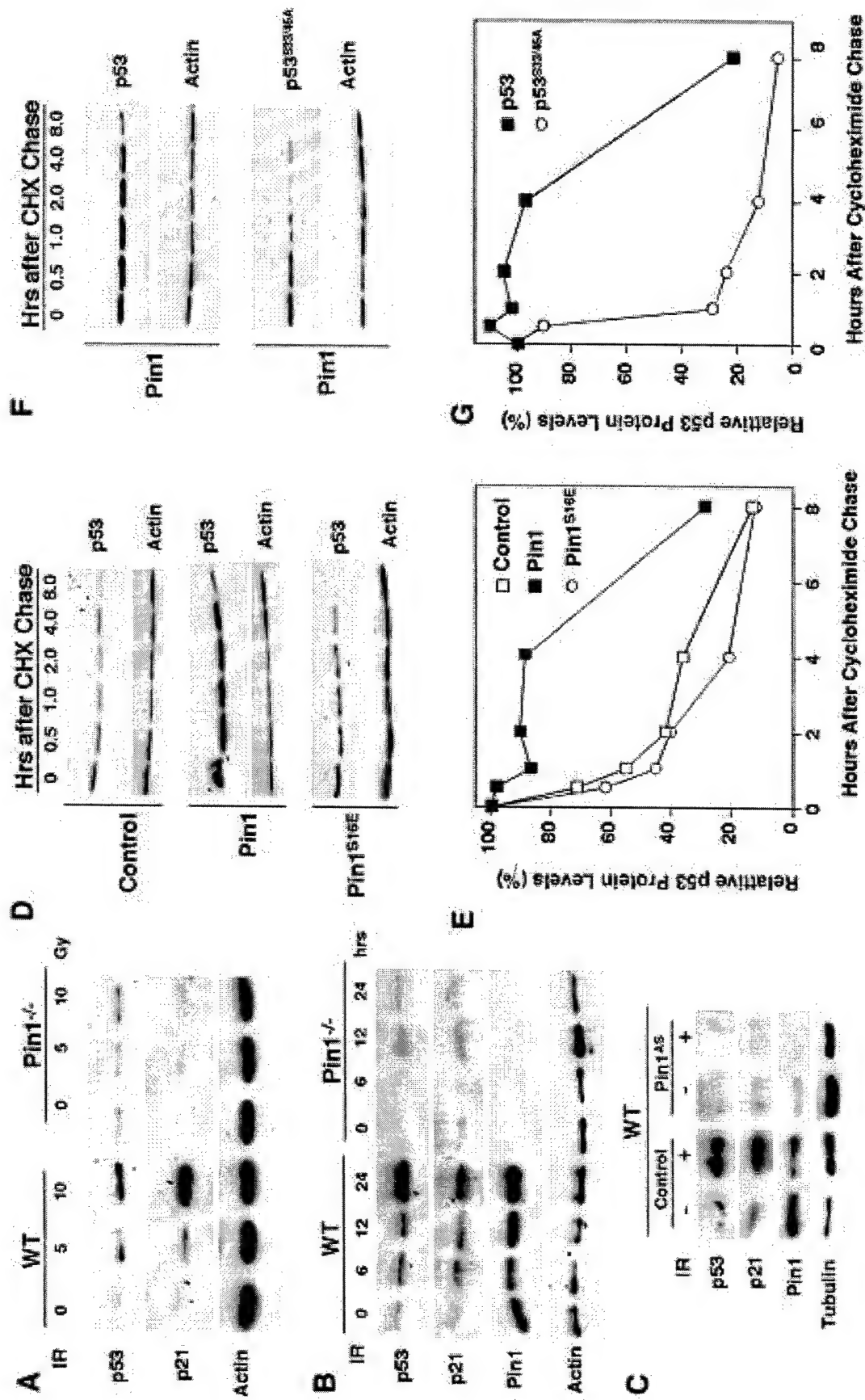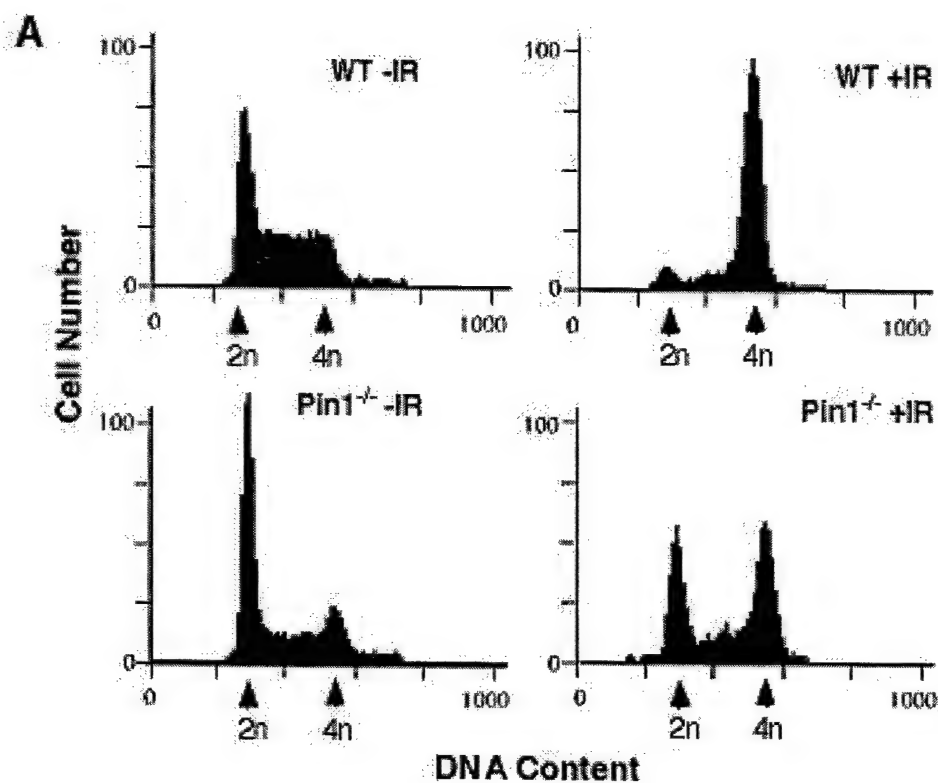
B

	-IR (%)				+IR (%)			
	sub G1	G1	S	G2/M	sub G1	G1	S	G2/M
WT MEFs	0	42.5	35.4	19.9	0	5.9	8.3	84.9
Pin1⁻/⁻ MEFs	0	53.9	20.4	22.9	1.2	26.0	20.7	50.0

Wulf et al. Fig. 4

Telomeric protein Pin2/TRF1 induces mitotic entry and apoptosis in cells with short telomeres and is down-regulated in human breast tumors

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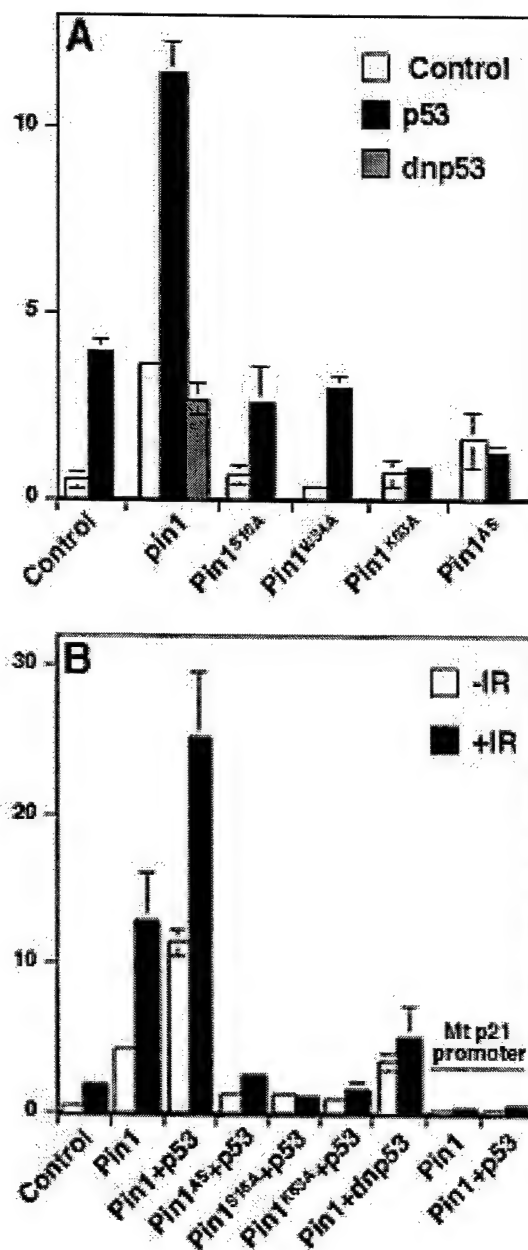[1]Cancer Biology Program, Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, MA 02215, USA

Telomeres are essential for cell survival and have been implicated in the mitotic control. The telomeric protein Pin2/TRF1 controls telomere elongation and its expression is tightly regulated during cell cycle. We previously reported that overexpression of Pin2/TRF1 affects mitotic progression. However, the role of Pin2/TRF1 at the interface between cell division and cell survival remains to be determined. Here we show that overexpression of Pin2 induced apoptosis in cells containing short telomeres, but not in cells with long telomeres. Furthermore, before entering apoptosis, Pin2-expressing cells first accumulated in mitosis and strongly stained with the mitosis-specific MPM2 antibody. Moreover, Pin2-induced apoptosis is potentiated by arresting cells in mitosis, but suppressed by accumulating cells in G1. In addition, overexpression of Pin2 also resulted in activation of caspase-3, and its proapoptotic activity was significantly reduced by inhibition of caspase-3. These results indicate that up-regulation of Pin2/TRF1 can specifically induce entry into mitosis and apoptosis, likely via a mechanism related to activation of caspase-3. Significantly, we also found that, out of 51 human breast cancer tissues and 10 normal controls examined, protein levels of Pin2/TRF1 in tumors were significantly lower than in normal tissues, as detected by immunoblotting analysis and immunocytochemistry. Since down-regulation of Pin2/TRF1 allows cells to maintain long telomeres, these results suggest that down-regulation of Pin2/TRF1 may be important for cancer cells to extend their proliferative potential. *Oncogene* (2001) **20**, 1497–1508.

Keywords: apoptosis; cancer; cell cycle; pin2/TRF1; telomeres; telomeric protein

Introduction

Telomeres are essential for preserving chromosome integrity during cell division. Telomeres are composed

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of repetitive DNA sequences of TTAGGG arrays concealed by a complex of telomeric proteins that protects the ends from exonucleolytic attack, fusion and incomplete replication (Greider and Blackburn, 1996; Lundblad, 2000; Zakian, 1995). There is growing evidence suggesting that both the shielding of telomeric ends and their elongation are dependent on telomere-binding proteins. For example, homeostasis of telomere length in budding and fission yeast cells requires the telomeric proteins Rap1p and Taz1, respectively (Cooper *et al.*, 1997; Krauskopf and Blackburn, 1996; Marcand *et al.*, 1997). Mutagenesis analyses of telomeric sequences of *Kluyveromyces lactis* also suggest that telomere length is modulated by proteins that bind to double strand telomeric DNA (McEachern and Blackburn, 1995). Telomere maintenance in mammalian cells is also regulated by telomere binding proteins, including TRF1 and TRF2 (Chong *et al.*, 1995; van Steensel and de Lange, 1997). TRF1 has been shown to negatively regulate telomere maintenance; overexpression of TRF1 accelerates telomere shortening, whereas dominant-negative TRF1 increases telomere elongation (van Steensel and de Lange, 1997). These results indicate that telomere-binding proteins play a pivotal role in telomere metabolism.

Several observations link telomeres to mitotic progression. In *Drosophila*, deletion of telomeres triggers mitotic arrest and apoptosis (Ahmad and Golic, 1999). In fission yeast, telomeres are clustered at the nuclear periphery in G2, but this association is disrupted in mitosis (Funabiki *et al.*, 1993), and telomeres have been shown to mediate the attachment of chromosomes to spindle bodies and lead chromosome movement in meiotic prophase (Chikashige *et al.*, 1994). In budding yeast, elimination of telomeres causes a Rad9p-mediated cell cycle arrest in G2 in budding yeast (Sandell and Zakian, 1993) and mutations in the related *TEL1* and MEC1 genes result in shortened telomeres, G2/M checkpoint defect and genomic instability (Greenwell *et al.*, 1995; Sanchez *et al.*, 1996). Similarly, mutations in its human counterpart, the *ATM* gene, causes ataxia-telangiectasia (AT) both in humans and mice, displaying a wide range of abnormalities, including those related to telomere dysfunction (Barlow *et al.*, 1996; Elson *et al.*, 1996; Savitsky *et al.*, 1995; Xu *et al.*, 1996; Xu and Baltimore, 1996). More interestingly, cell lines derived from AT patients have

shortened telomere lengths (Metcalfe *et al.*, 1996; Pandita *et al.*, 1995; Xia *et al.*, 1996) and defected G2/M checkpoint (Beamish *et al.*, 1994; Rudolph and Latt, 1989). Finally, mutations in the *Tetrahymena* telomeric DNA sequence has been shown to cause a block in anaphase chromosome separation (Kirk *et al.*, 1997). Collectively, these results suggest that telomeres may be important for regulation of mitosis. However, little is known about the identity and function of the signaling molecule(s) involved in this process.

We independently isolated the telomere-binding protein Pin2 as a one of three proteins, Pin1–3, which interact with NIMA kinase that is an essential mitotic kinase in Aspergillus nidulans (Lu *et al.*, 1996). Characterization of these Pin proteins shows that they are all involved in mitotic regulation (Lu, 2000). Pin1 binds and regulates the function of a subset of phosphoproteins by controlling the conformation of specific phosphorylated Ser/Thr-Pro motifs (Lu *et al.*, 1999a,b; Shen *et al.*, 1998; Yaffe *et al.*, 1997; Zhou *et al.*, 2000). Pin2 is identical in the sequence to TRF1 apart from an internal deletion of 20 amino acids (Shen *et al.*, 1997). TRF1 and Pin2 are likely two alternatively spliced isoforms of the same gene *PIN2/TRF1*, as suggested by Young *et al.* (1997). For clarity, we will here use Pin2 for the 20 amino acid deletion isoform and TRF1 for the 20 amino acid containing isoform, as they were originally identified (Chong *et al.*, 1995; Shen *et al.* 1997), and will refer to the endogenous Pin2 and TRF1 proteins as Pin2/TRF1 since it is difficult to physically or functionally separate these isoforms at the present time. However, we have shown that Pin2 is 5–10-fold more abundant than TRF1 in the cells and the expression level of Pin2/TRF1 is tightly regulated during the cell cycle (Shen *et al.*, 1997). Both Pin2 and TRF1 contain a D-like motif similar to the destruction box present in many mitotic proteins, and their protein levels are significantly increased in late G2 and mitosis and then degraded as cells exit from mitosis. Furthermore, overexpression of Pin2 or TRF1 resulted in accumulation of the cells in G2 or M phase of the cell cycle (Shen *et al.*, 1997). Although these results together suggest that Pin2/TRF1 may affect mitotic progression, it is not clear whether Pin2/TRF1 has any specific effect on mitosis. Furthermore, it is unknown why overexpression of Pin2/TRF1 has no effect on the cell cycle in some cells (van Steensel and de Lange, 1997).

Here, we describe that overexpression of Pin2 induced entry into mitosis and apoptosis only in cells with short telomeres, but not in cells with long telomeres. Moreover, Pin2-induced apoptosis is potentiated by mitotic arrest, but not suppressed by G1 arrest. Importantly, Pin2 overexpression induced activation of caspase-3, a key executioner of apoptosis, and its ability to induce apoptosis was significantly suppressed by inhibition of caspase-3. These results have demonstrated that Pin2/TRF1 can specifically affect the cell cycle, triggering mitotic entry and apoptosis in the cells containing short telomeres. Consistent with a potential role of Pin2/TRF1 in regulation of cell survival, we found that protein levels of Pin2/TRF1 were significantly reduced in human breast cancer tissues, as compared with those in normal controls. Since down-regulation of Pin2/TRF1 allows cells to maintain long telomeres (van Steensel and de Lange, 1997), these results suggest that down-regulation of Pin2/TRF1 may be important for cancer cells to divide continuously.

Results

Overexpression of Pin2 induces apoptosis in HeLa cells

We previously showed that ectopic expression of Pin2 or TRF1 in HeLa cells caused accumulation of cells with 4N DNA content (Shen *et al.*, 1997), which is normally observed in G2 and/or M phase of the cell cycle. To further characterize this Pin2/TRF1-induced phenotype, we transfected cDNA constructs for Pin2 and TRF1 into HeLa cells and A-T22IJE-T. A-T22IJE-T cells were originally derived from primary A-T fibroblasts and contain no ATM protein (Ziv *et al.*, 1989). After transient expression, two proteins were detected in the nucleus of cells 8 h after transfection by indirect immunofluorescence. However, cells expressing Pin2 or TRF1 rounded up and died after 28–32 h, and a few cells were detectable at 72 h, suggesting that Pin2 and TRF1 were toxic to the cells. To visualize surviving Pin2/TRF1-expressing cells, we co-transfected cells with a β-gal expression construct. After co-transfection of HeLa or A-T22IJE-T cells with Pin2 or TRF1 and β-gal constructs, the number of X-gal-stained surviving blue cells was significantly reduced, as compared with that of empty vector- or antisense Pin2-transfected cells (Figure 1, data not shown). Since there was no detectable difference in phenotypic changes induced by Pin2 or TRF1 (Table 1), we focused on Pin2 for subsequent experiments.

The morphological changes of Pin2-expressing cells were suggestive of apoptosis. To confirm that overexpression of Pin2 indeed induces apoptosis, we first used the TUNEL assay, which detects apoptosis-specific DNA breaks (Douglas *et al.*, 1998; Gavrieli *et al.*, 1992). To detect transfected cells by flow cytometry, HeLa cells were co-transfected with CD20, as described (Zhu *et al.*, 1993). Whereas there were less than 0.4–1.0% TUNEL-positive cells in vector-transfected cells, about 15–20% of TUNEL-positive cells were detected in a Pin2-transfected cell population (data not shown). To directly observe morphological changes of Pin2-expressing cells, we inserted green fluorescence protein (GFP) at the NH_2-terminal end of the Pin2 cDNA. After 28 h transfection, 38% of GFP-Pin2-expressing HeLa cells and 56% of GFP-Pin2-expressing A-T22IJE-T rounded up and the condensed chromatin became fragmented, with the formation of micronuclei (Figure 2a, also see 4a), which are characteristic of apoptotic cells. When Pin2-transfected cells were subjected to flow cytometry after staining with propidium iodide, as shown later in Figure 5b,

A

| Vector | Pin2 |

0.5 µg

1.0 µg

2.0 µg

B
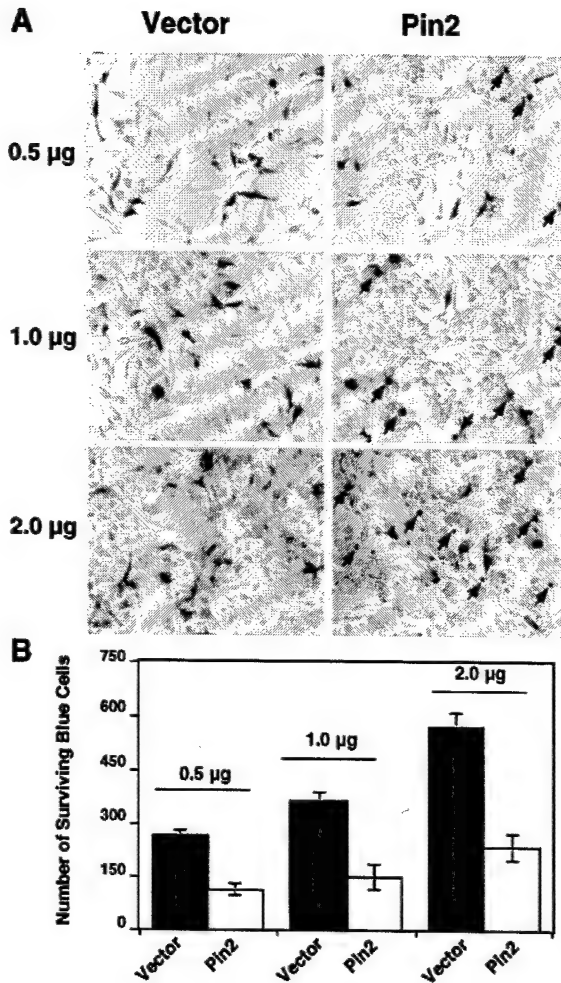
Figure 1 Expression of Pin2/TRF1 affects cell viability in a concentration-dependent manner. HeLa cells were cotransfected with different amounts of the Pin2/TRF1 expression construct or control vector, together a pSV2-lacZ reporter construct. Cells were fixed at 60 h after transfection, stained with X-gal and examined microscopically (a). Arrows point to dead cells. The number of surviving blue cells were counted and presented in (b)

about 25% of GFP-Pin2-expressing HeLa cells contained a sub-G1 DNA content, which is another feature of apoptosis. These multiple assays confirmed that ectopic expression of Pin2 can induce apoptosis in HeLa and A-T22IJE-T cells.
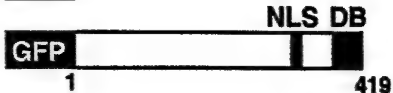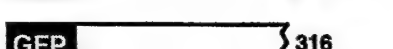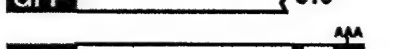
The ability of Pin2 to induce apoptosis depends on the concentration of unbound Pin2 and telomere length in the cell

After establishing that Pin2/TRF1 induces apoptosis, we examined its relationship to telomeres. Since the C-terminal Myb-type DNA-binding domain of Pin2/TRF1 binds telomeric DNA repeats and is also required for Pin2/TRF1 to inhibit telomere elongation

and to affect mitotic progression (Shen *et al.*, 1997; van Steensel and de Lange, 1997), we examined whether this domain is required for Pin2 to induce apoptosis. Although two C-terminal truncation mutants, Pin2$^{1-372}$ and Pin2$^{1-316}$ were expressed in cells at levels that were similar to that of wild-type protein, they were not localized at telomeres (Figure 2), as shown previously (Shen *et al.*, 1997; van Steensel and de Lange, 1997). In contrast to wild-type Pin2, neither of the truncation mutants induced apoptosis in HeLa (data not shown) or A-T22IJE-T cells and less than 10% of transfected cells were apoptotic (Figure 2a–c). These results indicate that the full-length Pin2, including telomeric DNA-binding domain is required for apoptosis induction. To further examine whether the ability of Pin2 to induce apoptosis depends on the actual binding of the full-length Pin2 to the telomeric DNA, we introduced triple Ala substitutions into three residues in Pin2, Lys401, Asp402 and Arg403. These three residues are highly conserved in Myb-type DNA-binding domains of telomeric proteins and are also involved in binding telomeric DNA, as revealed by determining the crystal structure of the DNA-binding domain of yeast RAP1 in complex with telomeric DNA (Konig *et al.*, 1996). When the triple Pin2 mutant (Pin2^{3A}) expression construct was transfected into HeLa cells, the mutant protein was detected in the nucleus, but not at telomeres (Table 1), as expected. However, Pin2^{3A} still induced apoptosis (Table 1). These results indicate that apoptosis is induced by the full-length Pin2 protein, but its telomeric binding is not required, suggesting that apoptosis is likely due to a high concentration of unbound Pin2.

Since apoptosis induced by inhibition of telomerase depends on telomere length (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Zhang *et al.*, 1999), we asked whether telomere length affected the ability of Pin2 to induce apoptosis. To address this question, we compared the ability of Pin2 to induce apoptosis in six cell lines with different telomere lengths, including HeLa1.2.11, which is derived from HeLa cells. In contrast to most HeLa cells that contain short telomeres (1–3 kb), HeLa1.2.11 cells have rather long telomeres (15–30 kb) (Table 1) (Ishibashi and Lippard, 1998). Interestingly, Pin2 potently induced the apoptotic phenotype in three different cell lines, HeLa, A-T22IJE-T and A431, all of which contain short telomeres (Figure 3a, Table 2) (Metcalfe *et al.*, 1996; Pandita *et al.*, 1995; Xia *et al.*, 1996; Zhang *et al.*, 1999). In contrast, Pin2 almost completely failed to induce significant apoptosis in three other cell lines, HeLa1.2.11 and 293 and HT1080, which contain long telomeres (Table 2) (Ishibashi and Lippard, 1998; van Steensel and de Lange, 1997; Zhang *et al.*, 1999). For example, in HeLa1.2.11 cells, expressed GFP-Pin2 was highly concentrated at telomeres, displaying a very prominent speckled pattern without affecting cell viability (Figure 3b), which is consistent with the fact that these cells have long telomeres (Ishibashi and Lippard, 1998). These results show that the ability of Pin2 to induce apoptosis at least in part depends on

Table 1 Induction of apoptosis by Pin2 and its mutants

Pin2 construct		Telomeric binding	Apoptosis (%)
Vector	GFP	-	5.4±2.2
Pin2	GFP NLS DB 1 419	+	37.6±8.9
Pin2$^{1-372}$	GFP 372	-	6.8±2.4
Pin2$^{1-316}$	GFP 316	-	6.2±1.9
Pin2^{3A}	GFP AAA	-	34.3±7.3
TRF1	GFP 439	+	32.3±5.6

Expression constructs expressing GFP-Pin2 or TRF1 or its various mutants were transfected into HeLa cells. Cells were fixed at 28 h after transfection, and stained with DAPI, followed by determining percentage of cells displaying apoptotic phenotype in at least 300 GFP-positive cells. The results represent the mean±s.d. of at least three experiments. The telomeric binding of TRF1, Pin2 and its truncation mutants was reported previously (Shen et al., 1997) and the same method was used to determine the telomeric binding of Pin2^{3A}. NH$_2$-terminal GFP box, GFP epitope tag inserted at the NH2-terminus; DB, the Myb-type DNA-binding domain that binds the telomeric DNA repeats; NLS, nuclear localization signal; I, 20 amino acid insert unique to TRF1
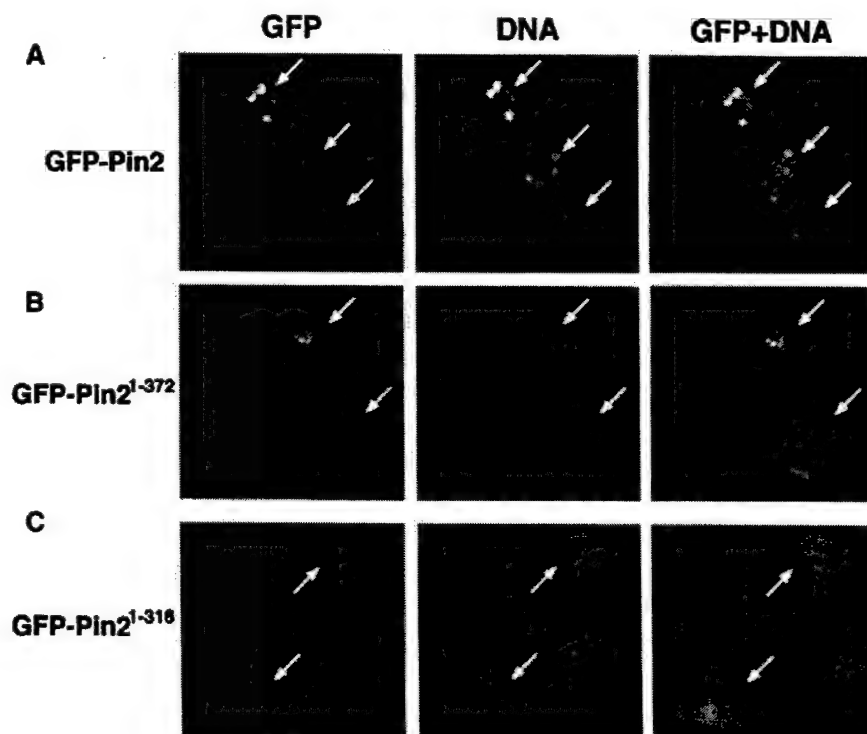
Figure 2 Expression of Pin2, but its telomere-binding mutants, induces apoptosis. A-T22IJE-T and HeLa cells were transfected with expression constructs encoding GFP-Pin2 (a) or its COOH-terminal truncation mutants, GFP- Pin2$^{1-372}$ (b) or - Pin2$^{1-316}$ (c). Cells were fixed at 30 h after transfection and stained with DAPI. Similar results were also obtained in HeLa cells (not shown). Arrows point to transfected cells. GFP alone did not induce apoptosis (not shown)
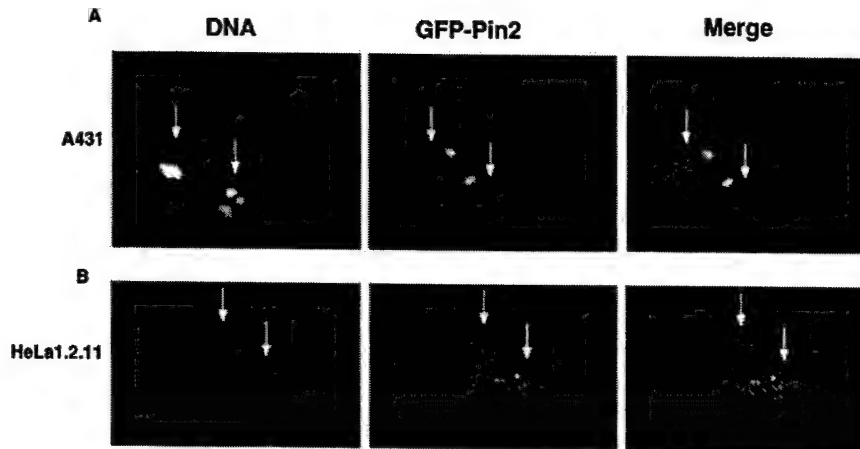
Figure 3 Expression of Pin2 induces apoptosis in A431 cells, but not in HeLa1.2.11 cells. A431 (**a**) and HeLa1.2.11 (**b**) cells were transfected with a GFP-Pin2 construct or the control GFP vector for 28 h, then fixed and stained with DAPI, followed by microscopy. Arrows point to Pin2-expressing cells. GFP alone did not induce apoptosis (data not shown)

Table 2 Pin2 induces apoptosis in cells with short telomeres, but not in cells with long telomeres

Cell line	Telomere length	Pin2-induced apoptosis
HeLa	Short (1–3 kb)[a]	+
A-T22IJE-T	Short (1–4 kb)[b]	+
A431	Short (1–3 kb)[c]	+
HeLa1.2.11	Long (15–30 kb)[d]	−
293	Long (6–12 kb)[c]	−
HT1080	Long (4–9 kb)[e]	−

Various cell lines were transfected with GFP-Pin2 expression construct or control GFP vector for 30 h and then fixed and stained with DAPI, followed by determining percentage of cells displaying apoptotic phenotype in total GFP-positive cells. [a]Telomere length in HeLa cells used was about 1–3 kb, determined as described in Materials and methods (data not shown); [b]Telomere length in all A–T cell lines examined is reported to be 1–4 kb (Pandita *et al.*, 1995; Xia *et al.*, 1996; Metcalfe *et al.*, 1996), although telomere length in A-T22IJE-T cell line has not been specifically determined; [c]Telomere length in A431 and 293 is reported to be 1–3 and 10–12 kb respectively (Zhang *et al.*, 1999); [d]Telomere length in HeLa1.2.11 is reported to be 15–30 kb (Ishibashi and Lippard, 1998); [e]Telomere length in HT1080 is reported to be 4–9 kb (van Steensel and de Lange, 1997); +, Apoptotic phenotype is easily detected in 30–55% of GFP-Pin2-expressing cells; −, Apoptotic phenotype is detected below 5–12% of GFP-Pin2-expressing cells, which were similar to control GFP-expressing cells

telomere length. These results are also consistent with our findings that the ability of Pin2 to induce apoptosis depends on the concentration of unbound Pin2.

Overexpression of Pin2 induces mitotic entry and apoptosis

To examine whether Pin2 specifically affects cell cycle progression before inducing apoptosis, we performed a detailed time course analysis of morphological changes of Pin2-transfected cells. At 12–16 h after transfection, GFP-Pin2 was primarily localized in the nucleus with punctate speckles during interphase, and was concentrated at telomeres with some diffuse staining all over the cells in the mitotic stage (Figure 4a). These patterns of the localization were similar to those of endogenous Pin2/TRF1 (Shen *et al.*, 1997; van Steensel and de Lange, 1997), indicating that GFP does not affect the localization of the Pin2 proteins. Importantly, the number of mitotic cells was significantly increased in the population of GFP-Pin2-expressing cells during the period of 20–24 h after transfection (Figure 4a,b). However, these Pin2-transfected cells apparently did not progress through normal mitosis. Instead, the condensed chromatin eventually seemed to become fragmented and micronuclei were formed (Figure 4a,b), a phenotype characteristic of apoptotic cells. These results suggest that ectopic expression of Pin2 leads to entry into mitosis, which is followed by apoptosis.

If Pin2 first induces mitotic entry and then apoptosis, there are at least two predictions. First, Pin2-induced apoptotic cells would have some mitosis-specific markers and, second, Pin2-induced apoptosis would be increased if cells are arrested at mitosis by other approaches, but decreased if cells are not allowed to enter mitosis. To examine the first prediction, we stained Pin2-transfected cells with the phospho-specific MPM-2 monoclonal antibody because MPM-2 specifically recognizes a subset of mitosis-specific phosphoproteins and has been widely used as a maker for mitotic cells (Davis *et al.*, 1983; Matsumoto-Taniura *et al.*, 1996; Vandre *et al.*, 1986; Westendorf *et al.*, 1994; Yaffe *et al.*, 1997). As shown in Figure 4c, most Pin2-expressing cells were strongly stained with MPM-2 20–24 h after transfection. This staining was also observed in Pin2-expressing apoptotic cells even 28–32 h after transfection, although at a weaker intensity (Figure 4c). This, however, is expected because it is impossible to maintain the high level of mitotic phosphorylation at such a stage of apoptosis. These results confirm that

A

B

C
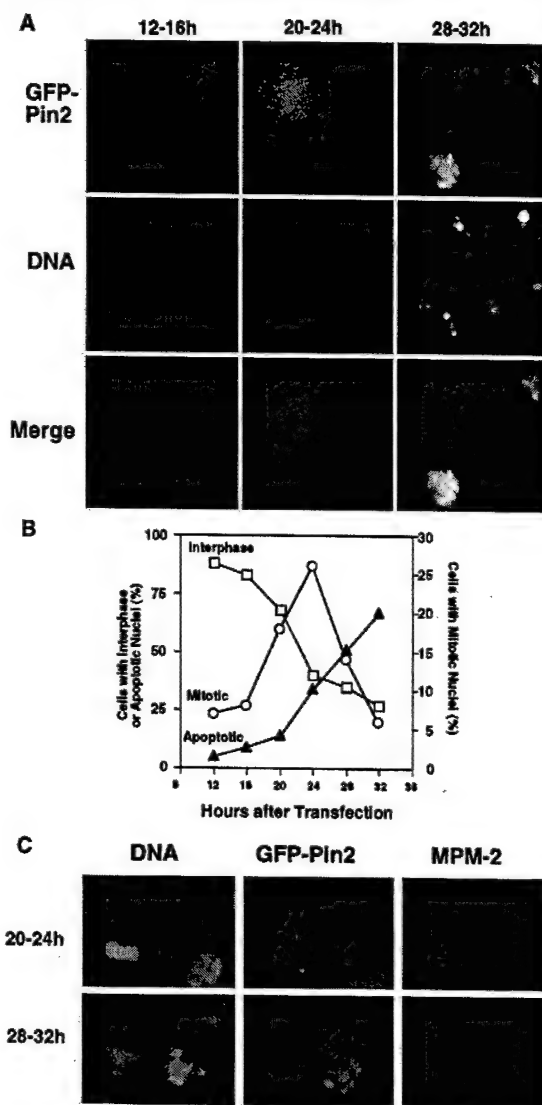
Figure 4 Expression of Pin2 induces mitotic entry and apoptosis in HeLa cells. After transfection with GFP-Pin2 construct, HeLa cells were monitored over time and fixed at times indicated, stained with DAPI and microscopically examined (a). The cells with interphase, mitotic or apoptotic nuclear morphology in all GFP-Pin2-expressing cells were counted (b). Cells were stained with MPM-2 antibody, followed by fluorescence microscopy (c)

Pin2-expressing cells are accumulated at mitosis before entering apoptosis.

To examine the second prediction, we transfected HeLa cells with GFP-Pin2 or membrane-localized control GFP (TM-GFP) for 12 h and then treated the cells with nocodazole or lovastatin, followed by analysing the cell cycle profiles of transfected and non-transfected cells using flow cytometry analysis. The membrane-localized TM-GFP was used instead of GFP because regular GFP leaked out of cells after fixing with ethanol for flow cytometric assay. When

cells were transfected with TM-GFP, both GFP-positive and -negative cells were almost completely arrested at mitosis after nocodazole treatment. Lovastatin treatment significantly increased G1 cells and reduced S phase cells, although the arrest was not complete (Figure 5a), as shown previously (Jakobisiak *et al.*, 1991; Keyomarsi *et al.*, 1991). This is likely due to the difficulty in completely synchronizing HeLa cells

A

B
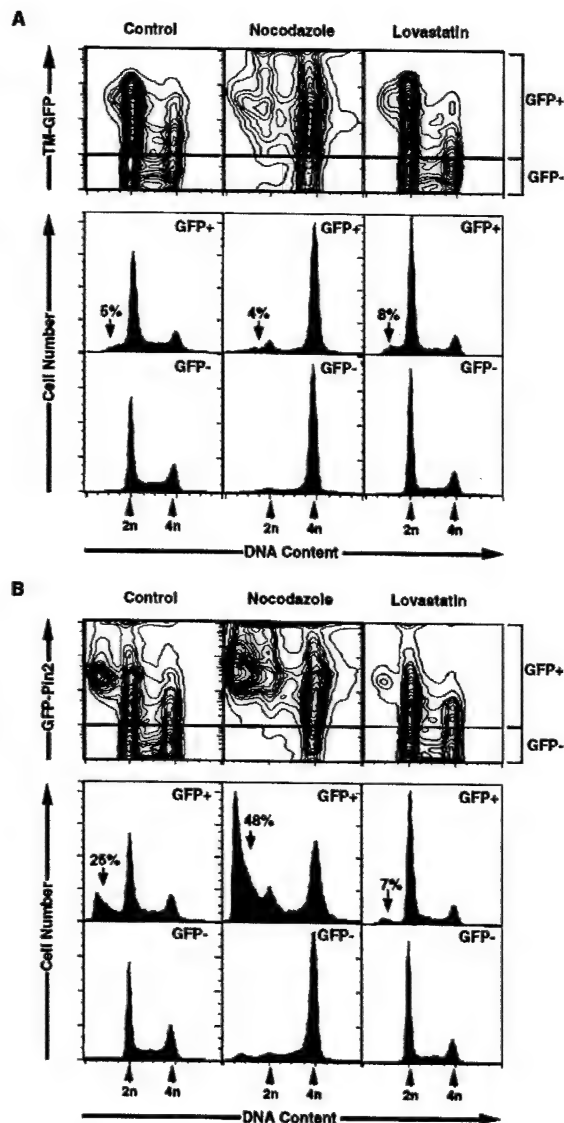
Figure 5 Pin2-induced apoptosis is potentiated by arresting cells at mitosis, but suppressed by arresting cells in G1. HeLa cells were transfected with membrane-localized TM-GFP vector (a) GFP-Pin2 (b) for 12 h and treated with 100 ng/ml nocodazole for 16 h or 20 μM lovastatin for 20 h to accumulate cells at mitosis or G1, respectively. Cells were stained with propidium iodide and GFP-positive and -negative cells, separated and their cell cycle profiles analysed using flow cytometry. Percentages and arrows indicate apoptotic cells with the sub-G1 DNA content in total cells examined

(Keyomarsi *et al.*, 1991). Furthermore, sub-G1 apoptotic cells were rather minimal (Figure 5a). These results indicate that nocodazole and lovastatin do produce the expected cell cycle arrests, which are not significantly affected by GFP. When the cells were transfected with GFP-Pin2, the cell cycle profiles of the non-transfected cells were similar to those of the control cells (Figure 5b, GFP−). However, in GFP-Pin2-positive cells, about 25% of cells were apoptotic, as indicated by the sub-G1 DNA content (Figure 5b, GFP+). This Pin2-induced apoptosis was reduced to 7% after lovastatin treatment, but dramatically increased to 48% after nocodazole treatment (Figure 5b). These results indicate that Pin2-induced apoptosis is dramatically increased if cells are arrested at mitosis, but decreased if cells are arrested in G1. Taken together, the above results indicate that overexpression of Pin2 induces entry into mitosis and apoptosis.

Overexpression of Pin2 results in activation of capase-3

After establishing that overexpression of Pin2 induces mitotic entry and apoptosis, we asked whether Pin2 activates caspase-3, because during apoptosis caspase-3 is a key executioner that is responsible either partially or totally for the proteolytic cleavage of many essential proteins (Cryns and Yuan, 1998). Activation of caspase-3 requires proteolytic processing of its inactive form into activated two subunits, p17 and p12 (Nicholson *et al.*, 1995). Since cleaved caspase-3 antibodies can detect the fragments of activated caspase-3, it is possible to examine the activity of caspase-3 in cells using flow cytometry, as shown previously (Belloc *et al.*, 2000). To examine the effect of overexpression of Pin2 on activation of caspase-3, we transfected cells either with GFP-Pin2 or membrane targeted TM-GFP for 30 h. Cells were stained with the cleaved caspase-3 antibodies and immunoreactivity of GFP-positive and -negative cells was determined by flow cytometry. As shown in Figure 6a, the fluorescence intensity of cleaved caspase-3 was significantly increased in cells expressing GFP-Pin2, as compared with that of GFP-negative cells in the same transfection population. In contrast, the fluorescence intensity of cleaved caspase-3 showed little difference between TM-GFP-positive and -negative cells. These results indicate that caspase-3 is specifically activated in GFP-Pin2-transfected cells. To examine the significance of the capase-3 activation, we used the caspase-3 inhibitor DEVD-CHO. As shown in Figure 6b, the caspase-3 inhibitor significantly suppressed the ability of Pin2 to induce apoptosis. These results indicate that Pin2 is able to activate caspase-3 and that caspase-3 is a downstream mediator of Pin2-induced apoptosis.

Expression of Pin2/TRF1 is down-regulated in human breast cancer samples

It has been previously shown that overexpression of TRF1 induces telomere shortening (van Steensel and de Lange, 1997). We show that overexpression of Pin2
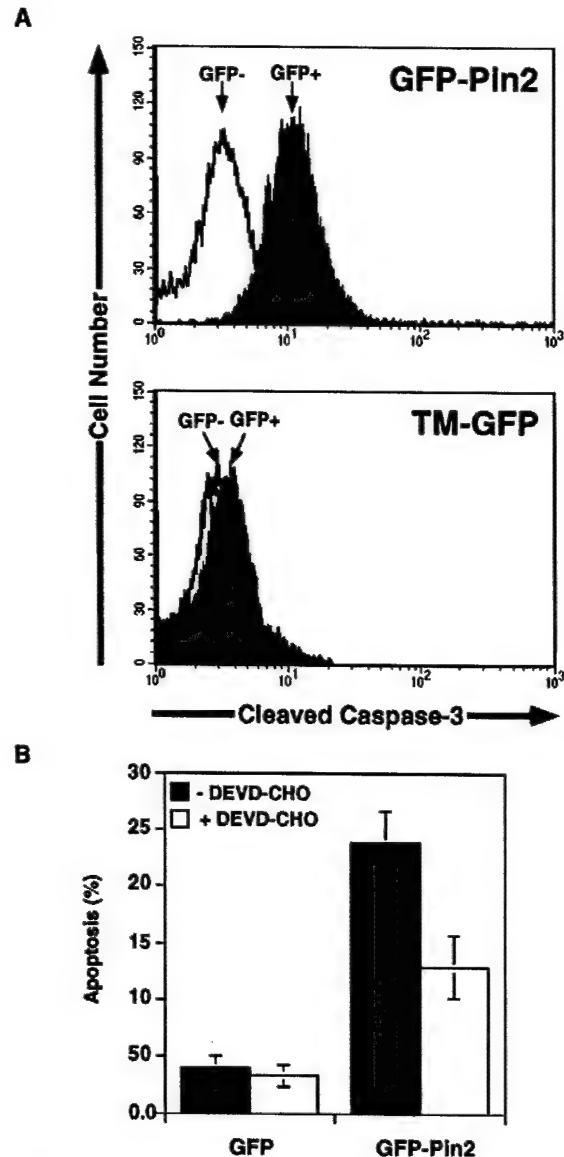
Figure 6 Overexpression of Pin2 leads activation of capase-3 and inhibition of capase-3 suppresses Pin2-induced apoptosis. (a) After 28 h of transfection with expression construct encoding GFP-Pin2 or the membrane targeted GFP (TM-GFP), HeLa cells were fixed and immunostained with cleaved caspase-3 antibodies and GFP-positive and -negative cells were sorted out and their immunoreactivity was determined by flow cytometry. Note, since regular GFP is leaked from cells after ethanol fixation, TM-GFP was instead used as a control. (b) Cells were pre-incubated with 10 μM of Ac-DEVD-CHO and then transfected with GFP-Pin2 or control GFP. Thirty hours later, cells were fixed and stained with DAPI, followed by determining percentage of apoptotic cells in total GFP-positive cells

or TRF1 induces apoptosis. These results led us to suspect that expression of Pin2/TRF1 may be reduced in cancer, where telomeres have to be maintained and apoptosis is often inhibited. To examine this possi-

bility, we compared protein levels of Pin2/TRF1 in normal human breast and breast cancer tissues using immunohistochemistry and immunoblotting with affi-

nity-purified anti-Pin2/TRF1 antibodies. Pin2/TRF1 was readily detected in ductal epithelial cells, connective tissue and blood vessels in normal breast tissues (Figure 7a). Furthermore, Pin2/TRF1 staining was primarily in the nucleus (Figure 7a), as shown previously (Shen et al., 1997; van Steensel and de Lange, 1997). However, infiltrating carcinoma cells displayed much weaker staining with the Pin2/TRF1 antibodies (Figure 7b). To ensure that these signals indeed represent Pin2/TRF1, the Pin2/TRF1-specific antibodies were depleted using GST-Pin2 beads prior to immunostaining. The Pin2/TRF1-depleted antibodies showed no immunoreactivity (data not shown), confirming the specificity of the antibodies, as described (Shen et al., 1997).

To confirm the immunostaining results, fresh normal or tumor breast tissues were ground in liquid nitrogen and lysates were directly subjected to immunoblotting analysis, followed by semi-quantification of protein levels using ImageQuant, as described (Lu et al., 1999a). As an internal control, we used actin, with the Pin2/TRF1 level in each sample being expressed as a ratio between Pin2/TRF1 and actin. Out of 10 normal and 51 primary human breast cancer tissues examined, we observed that levels of Pin2/TRF1 protein in all neoplastic breast tissues were significantly lower that those present in normal control tissues (Figure 7c, Table 3). Together, both immunostaining and immunoblotting analyses indicate that expression of *PIN2/TRF1* is significantly down-regulated in most breast cancer samples examined.

Discussion

We have demonstrated that up-regulation of Pin2 function results in mitotic entry and then apoptosis. Interestingly, this phenotype depends on the concentration of unbound Pin2 and on telomere length in the cells. Furthermore, overexpression of Pin2 leads to activation of caspase-3 and inhibition of caspase-3 significantly suppresses the ability of Pin2 to induce apoptosis. These results indicate that overexpression of Pin2 specifically induces mitotic entry and apoptosis

Figure 7 Down-regulation of Pin2/TRF1 expression in human breast cancer samples. (a, b) Immunostaining of Pin2/TRF1 in normal (a) and cancerous (b) human breast tissues. Fixed breast cancer sections were subjected to immunostaining with affinity-purified anti-Pin2/TRF1 antibodies. Magnification: 40×. (c) Comparison of Pin2/TRF1 levels in selected normal and cancerous human breast tissues. Breast tissues obtained from three normal and six breast cancer patients were powderized and the same amounts of total protein were directly separated on SDS-containing gels and transferred to membranes. The membranes were cut into two pieces and subjected to immunoblotting analysis using antibodies against to Pin2/TRF1 and actin, respectively

Table 3 Comparison of Pin2/TRF1 levels in normal and neoplastic breast tissues

	Number of cases	Age range	Pin2/TRF1 levels ($X \pm SD$)	p value
Normals	10	22–91	2.517 ± 1.635	0.0004
Tumors	51	28–90	0.835 ± 0.565	

The patient cohort included 47 invasive breast carcinoma and four ductal carcinoma *in situ*. Levels of Pin2/TRF1 in tissues were determined by immunoblotting analysis and semi-quantified using ImageQuant software, with the results being expressed as a ratio between Pin2/TRF1 and actin in each tissue. The significance of the differences in Pin2/TRF1 levels between normal controls and tumors was analysed using the Kruskall–Wallis test

likely via a mechanism that is related to telomere dysfunction. Finally, we found that levels of Pin2/TRF1 are significantly reduced in human breast cancer tissues, as compared with those in the normal control. Together with that down-regulation of Pin2/TRF1 allows cells to maintain long telomeres, these results suggest that down-regulation of Pin2/TRF1 may be important for the growth of cancer cells.

Several results support that the effects of exogenously expressed Pin2 are specific and related to telomeres shortening. First, Pin2-induced apoptosis depends on the concentration of unbound Pin2, which is likely to be higher in cells with short telomeres. Second, Pin2 induces apoptosis only in cells containing short telomeres, but not in cells containing long telomeres, similar to apoptosis induced by inhibition of telomerase (Hahn et al., 1999; Herbert et al., 1999; Zhang et al., 1999). Third, up-regulation of Pin2 leads to activation of caspase-3, a key executioner of apoptosis, that is responsible either partially or totally for the proteolytic cleavage of many essential proteins during apoptosis (Cryns and Yuan, 1998). Fourth, inhibition of caspase-3 suppresses the ability of Pin2 to induce apoptosis, indicating that activation of caspase-3 plays an important role during Pin2-induced apoptosis. These results argue that the ratio of 'free' and 'bound' Pin2 is crucial for cells, and increases as telomeres shorten, eventually leading to apoptotic cell death by mitotic catastrophe.

The findings that Pin2/TRF1 induces entry into mitosis and apoptosis are consistent with previous reports. Deletion of telomeres also induces mitotic arrest and apoptosis in *Drosophila* eyes *in vivo* (Ahmad and Golic, 1999). Furthermore, apoptosis is also triggered by inhibiting telomerase via expression of antisense nucleotide or dominant-negative mutants (Fu et al., 1999; Hahn et al., 1999; Herbert et al., 1999; Kondo et al., 1998a,b,c; Lee et al., 1998; Zhang et al., 1999) and inhibition of the telomeric protein TRF2 (Karlseder et al., 1999). These results consistently show that modulating telomere length can affect mitosis and lead to apoptosis. Furthermore, we have previously shown that the expression level of Pin2/TRF1 is cell cycle-dependent (Shen et al., 1997). Pin2/TRF1 is significantly increased when cells reach late G2 and M phase of the cell cycle, followed by degradation before cells exit from mitosis. Together with the findings that Pin2 contains a D-like motif similar to the destruction box present in many mitotic proteins, we have previously proposed that degradation of Pin2/TRF1 may be required for cells to exit from mitosis (Shen et al., 1997). Our current results, showing that overexpression of Pin2 leads to mitotic entry followed by apoptosis, further support the idea that the function of Pin2/TRF1 is tightly regulated during mitosis.

Pin2-induced apoptosis depends on telomere length in cells. Whereas Pin2 potently induces apoptosis in cells containing short telomeres, such as HeLa cells, A-T22IJE-T and A431, Pin2 fails to induce apoptosis in cells with long telomeres, such as 293, HT1080 and HeLa1.2.11, a HeLa subclone containing long telomeres, even though the protein is expressed and highly concentrated at long telomeres in these cells. This finding that the ability of Pin2 to induce apoptosis depends on telomere length may provide an explanation for why TRF1 has not been shown to induce apoptosis in some cells, including HT1080 cells (Karlseder et al., 1999; van Steensel and de Lange, 1997). It is also consistent with the recent demonstration that the ability of inhibiting telomerase to induce apoptosis highly depends on the length of telomeres (Zhang et al., 1999). Expression of dominant-negative telomerase mutants induces apoptosis only in cells that contain short telomeres, although it does not induce further shortening of telomeres (Zhang et al., 1999). Similarly, expression of Pin2 in those cells containing short telomeres does not further shorten telomeres (data not shown). Since telomere length is sensed by the concentration of bound telomeric proteins, as shown in the case of Rap1p (Marcand et al., 1997), a high concentration of bound Pin2/TRF1 in long telomere cells could be a signal that the telomeres are long enough for cells to continue dividing. Conversely, a high concentration of unbound Pin2/TRF1 in short telomere cells could indicate that the telomeres are too short for the cell to divide. This latter possibility is supported by our findings that the point mutant in the DNA-binding domain does not bind to the telomeric DNA but still potently induces apoptosis. Therefore, telomere length and the concentration of unbound Pin2 may be important signals for cell proliferation.

Although modulating the function of telomerase or either telomeric protein Pin2/TRF1 or TRF2 can all lead to apoptosis, the molecular pathways involved seem quite different. It has been demonstrated that apoptosis induced by inhibition of TRF2 is ATM- and p53-dependent (Karlseder et al., 1999). However, p53 is also functionally absent from all of cell lines sensitive to Pin2/TRF1-induced apoptosis in our studies due to the presence of HPV E6 or SV40 T antigen in these cells. Together with findings that Pin2 or TRF1 also potently induced apoptosis in ATM-negative A-T22IJE-T cells, these results indicate that Pin2/TRF1 induced apoptosis is ATM- and p53-independent. Similarly, p53 is also not required for apoptosis observed in telomerase-inhibited cells (Zhang et al., 1999). Given that both overexpression of Pin2/TRF1 and inhibition of telomerase inhibit telomere elongation (Hahn et al., 1999; Herbert et al., 1999; van Steensel and de Lange, 1997; Zhang et al., 1999), it is conceivable that Pin2/TRF1 may induce apoptosis via a mechanism similar to that of telomerase inhibition, although the actual signal to activate apoptosis remains to be elucidated. At least two different signaling pathways exist in telomere-mediated apoptosis. One is the ATM- and p53-dependent pathway that is activated by inhibition of TRF2 and the other is the ATM- and p53-independent pathway that is activated by inhibition of telomere elongation via up-regulating Pin2/TRF1 or inhibiting telomerase. The ability to pinpoint the induction of apoptosis in these two pathways may provide a powerful tool to investigate

the molecular nature of the apoptotic response to telomere dysfunction.

Although it remains to be determined whether Pin2/TRF1 is able to induce mitosis and apoptosis under physiological conditions, there are at least two pathological conditions where Pin2/TRF1-induced apoptosis may be important. One condition is the genetic disorder ataxia-telangiectasia caused by ATM mutations. These patients are hypersensitive to irradiation and cells derived from the patients contain short telomeres and display a prominent G2/M checkpoint defect upon irradiation. These ATM-negative cells fail to delay entry into mitosis and instead are prone to enter mitosis and apoptosis after irradiation (Metcalfe et al., 1996; Pandita et al., 1995; Smilenov et al., 1997; Xia et al., 1996). Significantly, the hypersensitivity to ionizing radiation is correlated with telomere loss (Metcalfe et al., 1996; Pandita et al., 1995; Smilenov et al., 1997; Xia et al., 1996). Interestingly, we have shown that ATM binds and negatively regulate the function of Pin2/TRF1 presumably via phosphorylation (Kishi et al, a manuscript submitted). More significantly, if the function of endogenous Pin2/TRF1 in ATM-negative cells is inhibited by stably expressing dominant-negative Pin2, cells are no longer sensitive to irradiation. Following irradiation, the Pin2/TRF1-inhibited A-T cells do not enter mitosis and apoptosis, but instead delay entry into mitosis, which is a normal DNA damage response for repairing damaged DNA. These results indicate that inhibition of endogenous Pin2/TRF1 function is sufficient to prevent DNA damage-induced mitosis and apoptosis, and also suggest that endogenous Pin2/TRF1 in ATM-negative cells is able to induce mitosis and apoptosis, at least upon DNA damage (Kishi et al., manuscript submitted). Another pathological condition where Pin2/TRF1-induced apoptosis may be significant is cancer cells. In contrast to most somatic cells, where telomeres are shortened with each cell division and there is a limited life span, cancer cells have an unlimited cell division potential and have to maintain their telomeres (Greider and Blackburn, 1996; Lundblad, 2000; Zakian, 1995). To maintain this continuous cell division, the function of Pin2/TRF1 is likely to be down-regulated in these cancer cells since up-regulation of Pin2/TRF1 results in telomere shortening, as shown previously (van Steensel and de Lange, 1997), and induces apoptosis, as shown here. Indeed, we have now found that Pin2/TRF1 is significantly down-regulated in most human breast cancer samples, as confirmed both by immunostaining and immunoblotting analysis. A recent immunohistochemical study also revealed a similar down-regulation of Pin2/TRF1 in gastrointestinal tumors (Aragona et al., 2000). Although the relationship between down-regulation of Pin2 and telomere length in tumor cells remains to be addressed, these results suggest that down-regulation of Pin2/TRF1 may be a general phenomenon in cancer and this down-regulation may allow cancer cells to extend their proliferative potential. Further studies on the role of Pin2/TRF1 in modulating cell proliferation and cell death may help understand the role of telomere maintenance in cellular aging and transformation.

Materials and methods

Transient transfection and apoptosis assays

For detecting apoptosis using β-gal assay, cells were co-transfected with pSV2-lacZ and vector encoding wild-type or mutant Pin2 for 48–60 h by using the Superfect reagents (Qiagen), fixed with 0.5% glutaraldehyde and stained with X-gal, as described (Kumar et al., 1994; Mayo et al., 1997). For the TUNEL assay, cells were cotransfected with Pin2 expression construct and the cell surface marker CD20 for 36 h and then stained with anti-CD20 antibody (Pharmingen), as described (Zhu et al., 1993). The stained cells were subjected to TUNEL staining and analysed by flow cytometry, as described (Douglas et al., 1998; Gavrieli et al., 1992). To directly observe the morphology of Pin2 expressing cells, Pin2 and its various mutants were expressed as C-terminal fusion proteins with GFP in cells (Clontech). Respective vectors were used in all transfections as controls. Transfected living cells were monitored over time and fixed at various time points. The indexes of interphase, mitotic and apoptotic cells were determined after staining the cells with the DNA-binding dye DAPI or the mitosis-specific monoclonal antibody MPM-2, as described (Lu and Hunter, 1995; Shen et al., 1997). The apoptosis rate was determined by counting about 300–400 GFP-positive cells.

Analysis of telomere restriction fragment length

Telomere restriction fragment length was determined, as described previously (van Steensel and de Lange, 1997). In brief, genomic DNA was isolated from the cultured cells using QIAamp Tissue Kit (QIAGEN), and digested with *Hinf*I and *RSA*I (New England Bio Labs) to generate the telomere restriction enzyme fragments. Ten μg of genomic DNA was separated on a 0.7% agarose gel. This gel was hybridized directly to a ^{32}P-labeled telomere probe, which was made with (AATCCC) primer using pSP73 Sty11 plasmid as a template in Klenow fragment.

Cell cycle analysis

To enrich cells in G1, cells were treated with 20 μM lovastatin for 20 h, as described (Jakobisiak et al., 1991; Keyomarsi et al., 1991). To block cells at mitosis, cells were incubated 100 ng/ml nocodazole for 16 h. For cell cycle analysis, cells were harvested by trypsinization, re-suspended in DMEM supplemented with 10% serum, washed in PBS, and then fixed in 70% ethanol. After washing cells once with PBS containing 1% BSA, DNA was stained with propidium iodide (10 μg/ml) containing 250 μg/ml of ribonuclease A, followed by flow cytometry analysis (Becton-Dickinson), as described (Lu and Hunter, 1995).

Flow cytometric analysis of caspase-3 activation and treatment of caspase-3 inhibitor

After 28 h of transfection with GFP-Pin2 or TM-GFP expression construct, HeLa cells were fixed and then immunostained with cleaved caspase-3 antibody (Cell Signaling Technology), followed by Rhodamine-conjugated anti-rabbit secondary antibodies, as described (Belloc et al., 2000).

The stained cells were analysed by flow cytometry for the detection of caspase-3 activation. To inhibit caspase-3, 10 μM of Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) was added to cells before transfection.

Expression of Pin2/TRF1 in human cancer tissues

Fifty-one cancerous and 10 normal breast tissue specimen were randomly selected. Tissue from the core of the tumor had been snap frozen in liquid nitrogen and powderized using a Microdismembrator (Braun). About 10 μg of the powderized tissues were re-suspended in 100 μl of SDS sample buffer. Immunoblotting analysis with anti-Pin2/TRF1 and anti-actin antibodies was performed as described (Shen *et al.*, 1997). Levels of Pin2/TRF1 were semi-quantified using Imagequant and the significance of the differences in Pin2/TRF1 levels between normal and cancer tissues was analysed, as described (Lu *et al.*, 1999a). To detect the localization of Pin2/TRF1 in human tissues, 50 μm sections were cut from breast cancer tissues, and then microwaved in an antigen retrieval buffer (Biogenex), as described by the manufacturer.

Endogenous peroxidase activity was blocked with H_2O_2, the sections were incubated with anti-Pin2/TRF1 antibodies that had been purified using GST-Pin2 glutathione beads (Shen *et al.*, 1997), and visualized by the immunoperoxidase staining protocol, as described (Lu *et al.*, 1999a).

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